AN EXPERIMENTAL INVESTIGATION OF MATERNAL-FOETAL F^{18} TRANSFER IN THE RAT

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A thesis embodying an original research programme
submitted by the undersigned as requirement
for admission to the degree of Master
of Dental Surgery within the
University of Sydney

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1967

PREFACE

The plan for this investigation was conceived with the help of Professor N.D. Martin, Head of the Department of Preventive Dentistry at the University of Sydney, whose advice and guidance throughout its implementation have been invaluable.

I am particularly grateful to Mr. P.D. Barnard of the Department of Preventive Dentistry for continual encouragement and generous assistance with the preparation and presentation of the results.

I should also like to express my thanks to Dr. P.C. Reade of the University of Adelaide for instruction in the Foetal Externalization Technique, to Mr. B.R. Gillings, Radiation Safety Officer in the Faculty of Dentistry for valuable advice and help with radiation equipment, to Miss Carol Dodd for technical assistance and to Mrs. E. Blears for clerical assistance.

The study was carried out at the University of Sydney using the facilities of the Department of Preventive Dentistry and the Institute of Dental Research. Animals were made available by Mr. J. Tye from the University of Sydney Animal House and Mrs. L. Rekai at the Institute of Dental Research. Radioactive fluoride was supplied by the Australian Atomic Energy Commission under the supervision of Mr. J. Robson. The work was supported by grants from the Australian Institute of Nuclear Science and Engineering, the University of Sydney and the Cancer Research Council of Australia.

Finally I wish to thank the members of my family who helped in many ways with the preparation of this thesis.

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INTRODUCTION

Exposure to an optimal concentration of fluoride continuously throughout the period of development of the permanent teeth is well recognized as an effective method of increasing resistance to dental caries. There is also increased resistance in the deciduous dentition but at what stage this protection is afforded the deciduous teeth has not been clearly established.

Since mineralization of the deciduous teeth commences in the human during the fourth month of pregnancy, theoretically maximum exposure to fluoride for the deciduous dentition necessitates exposure not only postnatally but also during the last six months of prental life. Consequently the provision of a fluoride dietary supplement for pregnant women drinking fluoride deficient water is indicated.

Both Erhardt [31] in 1874 and Crichton-Browne [26] in 1892 suggested prenatal fluoride supplements without valid scientific basis and it was almost fifty years before serious thought was given to this question.

Undoubtedly the most important consideration in relation to prenatal exposure to fluoride is whether or not dental caries resistance in the offspring is increased. Only clinical examination of the teeth of children who have had variable exposure to fluoride pre and postnatally and comparison of these with a similar group who have not been exposed to fluoride can finally determine what the benefit is.

Fluoride does pass the placenta at some stage during the period of gestation and is incorporated in the teeth. Its effects on the teeth depend on the amount that is taken up, which varies with the stage of development of the teeth and the quantity of fluoride that passes through the placenta. The degree of transfer depends, in turn, on the function of the placenta, the amount ingested and the maternal metabolism.

A partial answer to the problem can be obtained by studying the deposition of fluoride in the foetus. Technical difficulties arise in the detection of small amounts of fluoride but much of the evidence available has been obtained in this manner.

Attempts have been made to obtain the relationship between maternal and foetal blood fluoride content but the findings are somewhat conflicting. Few investigations have been really comprehensive because of differences in experimental technique, methods of analysis and species studied. However the transfer of fluoride has been clearly demonstrated by analysis of foetal and embryonic hard and soft tissues, maternal and cord blood and placental tissue and recently the use of the radio-active isotope F^{18}, has enabled its passage through the placenta to be directly traced in vivo.

The use of radioactive tracers provides an almost ideal situation for studying placental transfer under normal physiological conditions. Unfortunately it was not possible in this study to experiment with human subjects but by choosing a suitable laboratory animal a somewhat similar situation has been achieved.

The rat is an animal commonly used in dental research. It has a placenta that is, histologically at least, similar to the human placenta. Using the foetal externalization technique the animals can be injected with small amounts of radioactive fluoride under physiological conditions. The passage of minute amounts of the isotope can be traced by sampling blood and tissues or by whole body counting.

This study was designed to develop a suitable reproducible method of investigating placental transfer of fluoride. It aims to demonstrate, in the rat, the transfer of fluoride as F^{18}, to determine both the speed of initial transfer and the ensuing rate of transfer and to establish the relation between maternal, foetal and placental blood fluoride levels.

REVIEW OF LITERATURE

The presence of fluoride in blood and other body tissues was recognised early in the nineteenth century but its relation to mottled enamel was not established until extensive studies of this condition were undertaken in the United States of America in the early part of the twentieth century.

Dean [28] in 1933 proposed a classification of fluorosis when the relationship between fluorosis and dental caries had only been suggested. However, it was not until 1942 that epidemiological surveys in areas with varying water fluoride concentrations by Dean [30] finally established that ingestion of an optimal amount of fluoride during the period of tooth formation would provide a significant resistance to dental caries without any signs of fluorosis. This applied particularly to permanent teeth but it was also noticed by Dean [29] that the deciduous dentition of children who lived continuously in naturally fluoridated areas showed a reduction in dental caries.

Once the optimal level had been determined water fluoridation was introduced. The effect on the deciduous teeth has since been confirmed in fluoridated areas by Russell and White [90 91].

It is generally recognised that to obtain maximum caries inhibition in the permanent dentition exposure to an optimal level of fluoride must begin during the formative period [64 66 90]. Some studies indicate that maximum benefit can only be obtained when exposure occurs throughout the entire calcification period [66] or at least that caries inhibition is a function of the length of fluoride exposure prior to eruption [64 90]. Another study indicates that for pit and fissure surfaces exposure must begin in the early stages of calcification but that smooth surfaces can benefit fully when exposure begins two or three years before eruption [5]. This finding appears logical when it is considered that the smooth surface areas are probably the last formed. It has also been suggested that ...

maximum benefit can be obtained if fluoride consumption begins shortly before eruption in the pre-eruptive maturation stage [52 110].

The evidence seems to favour the idea that exposure should commence a considerable time before eruption.

If exposure throughout the whole or even a considerable part of the calcification period is necessary, then exposure to an optimal amount of fluoride must begin about six months prior to birth, when calcification of the deciduous teeth begins.

Exposure at this time depends entirely on the amount of fluoride ingested by the mother that reaches the foetus.

It is interesting to note that prenatal supplements of fluoride were first suggested by Erhardt [31] in 1874 and Crichton-Brown [26] in 1892 with considerable foresight but no valid scientific basis.

The possible value of prenatal fluoride was overlooked for some time because it was thought that the placenta acted as a barrier to fluoride. This theory tended to be confirmed by lack of evidence of fluorosis in the deciduous dentition.

The report by Smith and Smith [99] in 1935 of severe fluorosis in the deciduous teeth of children who drank water with a high fluoride content discounted the idea of a complete placental barrier. * In 1940 Day [27] substantiated this claim when he reported fluorosis of the deciduous teeth of children in India and indicated a belief in placental and mammary transfer of fluoride. Sognnaes [100] in 1941 also reported a condition suggestive of fluorosis in the deciduous teeth of the Tristan da Cunha people who had a high fish diet and 0.2 ppm fluoride in the water.

* These reports must be accepted with caution because of the lack of criteria at this time for the diagnosis of mottled enamel and possible confusion with non-fluoride opacities.

Roholm [89] found no evidence of fluorosis of the deciduous teeth despite its occurrence in the permanent teeth of children who had been nursed for long periods by fluorine intoxicated mothers. He believed that fluoride could not pass the placenta although he considered this to be an indication of mammary transfer.

Knowing now that at optimal levels fluorosis does not normally occur, the generally good condition of the primary teeth described by Roholm [89] could be an indication of at least a limited transfer. However, Roholm did suggest that species difference could occur, which accounted for specific changes found by Velu in the deciduous teeth of the horse.

Changes in the teeth of neonatal mice were observed by Fleming and Greenfield [41] after administration of fluoride to the mother during gestation. Alteration in ameloblasts and retarded enamel formation were noted.

Experimental fluorosis of the teeth due to prenatal fluoride has not otherwise been reported in animals although Cox et al [24 25] reported reduced caries incidence in the offspring when rats were given an adequate amount of fluoride (more than 20 ppm) during pregnancy. Moreover Shaw and Sognnaes [93] found a greater reduction in caries in the offspring of rats fed fluoride during pregnancy and lactation than if fluoride supplements were commenced later. On the other hand, Stookey et al [101] did not find any alteration in the incidence of caries in rats after the use of fluoridated water (25 ppm) during pregnancy.

Persistence for so long of the idea of the placental barrier was due to the fact that the field remained relatively unexplored because of the difficulty in the detection and accurate determination of small amounts of fluoride.

Today there are many reports of fluoride in foetal tissues but there is still doubt about the significance of prenatal fluoride. The only way of evaluating this is by comparing the teeth of children who have had both pre- and postnatal

fluoride with those who have had only postnatal fluoride or alternatively comparing a group of children who have had only prenatal fluoride with a group that has not been exposed to fluoride at any time

Carlos et al [18] examined small groups of children born before and after fluoridation in Newburg. He found little difference between children whose mothers drank fluoridated water during pregnancy and those who did not.

Blayney and Hill [11] reporting the results of the Evanston study found a reduction of about thirty per cent in the caries rate for the deciduous teeth of six, seven and eight year old children whose mothers used fluoridated water during pregnancy, compared with a base line control group. At least ninety per cent of the mothers had used the water throughout the entire gestation period. Comparison of this figure with the ten to seventeen per cent reduction found in a group of children who had fluoride from birth only, led the authors to conclude that the use of water containing 1.0 ppm of fluoride throughout the prenatal period, followed by its postnatal use gives greater protection to the deciduous teeth than when used only postnatally..

Carlos [17] has criticised this conclusion on the basis that it is not justified by the data published. He points out that fifty-three per cent of the mothers of the 1953 exposed-from-birth group had consumed fluoridated water at some time during pregnancy. This certainly confuses the issue but does not necessarily invalidate the conclusion drawn by Blayney and Hill [11].

Recent findings by Horowitz [60] are essentially in agreement with those of Carlos [18].

Feltman and Kosel [40] reported that children of women exposed to a prenatal dietary supplement had a higher caries incidence in the deciduous teeth than children who had also an additional postnatal supplement, but a lower caries incidence

than children who had no supplement at all. Feltman and Kosel stated further that the benefits were greater when the supplement was commenced during the first and second trimester than if used only in the third trimester.

Recently Tank and Storvick [104] found a higher caries prevalence in the deciduous teeth of children born before fluoridation that in those born after its introduction. Although not statistically significant because the sample with only postnatal exposure was rather small, this report tends to support the conclusions of Blayney and Hill [11]. Although the data on the efficiency of prenatal exposure to fluoride is inconclusive, the fact remains that wherever fluoridation is instituted fluoride is available to the foetus at some stage during prenatal life. For this reason a thorough knowledge of the mechanism of transfer is important. It is therefore necessary to understand the processes of absorption, storage and excretion which affect the concentration of fluoride in the blood reaching the placenta.

Absorption

Absorption of fluoride can occur from the lungs after inhalation, through the skin on handling or from the gastro-intestinal tract after ingestion. The last mentioned is the most common and of most interest.

Wallace-Durbin [109], Zipkin and Likins [117] and Wagner [107] have demonstrated, by analysis of the contents of the gastro-intestinal tract, that fluoride in soluble form is rapidly absorbed. Wallace-Durbin [109] reported the absorption of seventy-five per cent of an orally administered dose of radiofluoride from the rat stomach within an hour. Similar results were obtained by Zipkin and Likins [117] and Wagner [107]. Wagner found that the initial absorption rate was slow but a maximum rate was reached fifteen to twenty minutes after administration. At this time the rate of absorption was 80 per cent of the administered dose per hour.

In addition Ericsson [32] showed that 80 per cent of an oral dose was absorbed in eight hours by rats. Wagner's data [107] confirms this.

The absorption of fluoride even at low intake levels is dependent on the solubility of the compound ingested [77 111 117], and affected by the presence of other ions [108]. It was shown by Ericsson [32] that absorption of fluoride occurs to the same extent from milk as from water.

Further evidence indicating rapid absorption of fluoride can be demonstrated by its appearance in blood and urine after oral administration. In humans Carlson et al [19] found that the maximum plasma fluoride level was attained in one hour. The peak was reached in three quarters of an hour in rats [32], in three hours in sheep [85], and in five hours in cattle [85].

Upon entering the blood stream fluoride rapidly exchanges between plasma and red cells * to have a cell plasma ratio of approximately 0.4 in the blood of dogs [20], and in humans [19], and 0.54 in the blood of rats [109].

Blood Fluoride Levels

A variety of results has been presented relating normal human blood fluoride content to water-borne fluoride. The most reliable appear to be those of Singer and Armstrong [94] who developed an accurate method of determination using serum rather than whole blood, with the addition of magnesium oxide to prevent the loss of 50% or more fluoride during ashing.

Analysis by Armstrong and Singer [3] of the plasma of normal individuals indicates that while the level of fluoride in the water supply remains below 5.4 ppm the plasma fluoride level is maintained relatively constant at approximately 0.14 ppm by the homeostatic mechanism of the body and that only above 5.4 ppm is there any appreciable rise in blood fluoride. Singer and Armstrong [94] found plasma fluoride of 0.12 and 0.13 ppm in subjects ingesting water with fluoride content of 0.1 and 1.0 ppm respectively. In contrast to this, Yudkin et al [114] reported blood fluoride of 1.02

* Human blood constitutes 45% by vol. cells and 55% by vol. plasma.

and 1.36 ppm when the water supply contained 0.1 and 1.0 ppm. Values are presumably for whole blood.

Somewhat lower values were obtained by Smith et al [97]. Blood contained 0.01 and 0.04 ppm fluoride when the water supply contained 0.06 and 1.36 ppm. However, Gedalia et al [46] found blood fluoride values of the same order as those of Singer and Armstrong [94]. They reported 0.18 ppm in the blood of women where the water supply contained 0.55 ppm fluoride.

Despite the difference in absolute values quoted by these investigators, all studies tend to demonstrate a relatively stable blood fluoride content.

Gedalia et al [46 48] have also investigated blood fluoride in pregnant women. These results indicate that the level is slightly lower than in non-pregnant women but the significance of this cannot be evaluated without considering how much fluoride is present in the foetus at the same time.

In any case, Brzezinski et al [14] found similar quantities of fluoride in the blood of pregnant and non-pregnant rats but stressed the fact that 'this metabolism is different from that of pregnant women who tend to retain a fluoride surplus during pregnancy.'

Fluoride has been detected in the foetal circulation in humans and animals. The relationship between maternal and foetal levels will be considered in detail later in this review.

The same homeostatic mechanisms apparently control fluoride metabolism in animals as in man, although higher levels are required to increase blood fluoride of animals than are required by humans [14]. Despite an increase in blood fluoride with an increased exposure level [14 95], continued exposure to very high fluoride levels does not result in considerably higher blood fluoride [78 95 96]. Smith et al [96] found that exposure of dogs for four days to hydrogen fluoride at a pressure of 20 mg./mm^3 caused their blood fluoride to rise from a normal 0.0 - 0.13 ppm to 4.55 ppm but that despite continued exposure, this level could not be maintained and had fallen to 2.5 ppm in ten days.

A similar low blood fluoride content of 2.0 ppm was found by Minoguchi and Iwamoto [78] in dogs exposed to a dietary supplement of 30 to 40 mg. of sodium fluoride per kilogram of body weight for five months.

As already mentioned, the maximum plasma fluoride level occurs in humans one hour after oral administration. Carlson et al [19] calculated that not more than ten per cent of an ingested dose was present in the plasma at any one time.

The only instance of high plasma fluoride is after intravenous injection, but even then the clearance is rapid.

Wallace-Durbin [109], Ericsson and Malmnäs [34], Perkinson et al [85], Bell et al [9] and Bawden et al [6] have examined blood fluoride clearance in humans and animals after intravenous injection of radiofluoride. The same pattern of clearance was found in cattle, rats, rabbits, sheep and humans.

Ten minutes after injection no more than ten per cent of the total dose remained in the blood stream.

The elimination of fluoride from the blood takes place in three phases. The first and most rapid is attributed to mixing with body fluids. The second phase, which is longer, is skeletal uptake and the third urinary excretion.

Soft Tissue Distribution

High concentrations of fluoride are not found in soft tissues [21 57 103 109]. Tissue concentrations are related to blood fluoride [21] and decrease quickly as fluoride is removed from the circulating blood [57 109] to urine and skeleton. Only slight increases are noted with increases in intake [103].

The highest concentration is found in the kidney [57 103 109]. Carlson et al [21] found more fluoride in muscle tendon than other organs, not including the kidney, probably because the tendon is a potential site for calcified deposits [106 109]. High concentrations have been found in the placenta during pregnancy and this is related to areas of calcification [33 35 39].

Excretion and Deposition

The two principal mechanisms of removal of fluoride from the blood and tissue fluids are urinary excretion and skeletal deposition. When domestic waters are free of fluoride, the amount present in urine average 0.3 to 0.5 ppm [74]. Many foods contain traces of fluoride. McClure [75] estimates that the average diet provides 0.2 to 0.3 mg. of fluoride daily. A prompt elevation in urinary fluoride is found consistently after ingestion (or inhalation) of even small amounts of fluoride [74 116]. Zipkin and Leone [116] showed that after a single dose of 5 mg. of fluoride as sodium fluoride the most rapid elimination occurred during the first hour. After eight hours the rate had fallen to a normal 0.1 mg. per hour.

For very soluble fluorides, increases in urinary fluoride are proportional to the levels of fluoride intake [67 74 97].

For poorly soluble fluorides urinary fluoride is related to the amount absorbed rather than that ingested [67].

Gedalia et al [48] found that in pregnant women the urinary fluoride decreased monthly from the normal adult value in the fourth month to less than half of this quantity in the eighth month and did not return to normal until several months after delivery.

When the quantity of fluoride absorbed is not more than four to five mg. per day almost complete elimination by urine and perspiration occurs [77].

After prolonged ingestion however, skeletal storage takes place and the concentration in bones increases [51 69 76 119].

The increase in skeletal content is relatively linear with an increase in the drinking water up to 4.0 ppm [89 119].

Experimental work indicates that in rats and cattle storage increases with increase in exposure level and increase in time [81 89 103].

In rats Muhler [81] showed that the skeletal fluoride concentration continued to increase when the diet contained 8 ppm fluoride, whereas at 3 ppm storage did not occur. In cattle also storage increases with increased exposure [103].

The maturity of the skeleton evidently influences its capacity to retain fluoride [44] [118]. It has been found that in adult humans after introduction of fluoridation urinary fluoride concentrations become approximately equal to water levels in one week, while for children the period required is two to three years [118].

A similar situation evidently exists in rats. The work of Savchuck and Armstrong [92] and Wallace-Durbin [109] demonstrates a greater accumulation in young animals than in adults. After feeding young and mature rats water containing 20 ppm fluoride for sixty days the skeleton of the young rats retained 43 per cent of the total intake whilst the mature rats retained only 29 per cent [92].

Wallace-Durbin [109] found that a high portion of any intravenous dose of F^{18} was deposited in the skeleton in two hours.

It was found that the skeleton of young adult rats accumulated more F^{18} than did the skeleton of mature rats. The pattern of uptake, rapidly rising to a peak at four hours then slowly declining, was the same regardless of age or amount of fluoride administered, but nine hours later the skeletons of the young rats retained 56 per cent of the administered dose whilst the mature skeletons retained only 48 per cent.

Evidently the vascularity of the bone influences the storage, since Wallace-Durbin [109], Perkinson et al [85] and Volker et al [106] found the greatest fluoride concentration in the region of the epiphyseal cartilage, the most recently calcified bone.

Very high concentrations of fluoride have been found in the bones of persons exposed to high concentrations of fluoride for a considerable time [89]. Values as high as 13.1 mg/gm were obtained. The fluoride content was highest in the bones of individuals who had been exposed longest.

It has been suggested that a 'steady state' is reached when no further storage occurs despite continued exposure. This has been demonstrated by Savchuck and Armstrong [92] and is substantiated by the in-vitro work of Neuman et al [83].

Fluoride Deposition in Teeth

Initial deposition of fluoride in teeth occurs rapidly [35 85 109].

The peak concentration of radio fluoride occurred in rat incisors four hours after intravenous injection and in molars one hour after injection in Wallace-Durbin's study [109]. There was then a gradual decline. The uptake was lower in mature rats than in young adults.

The uptake of fluoride by the teeth depends on the level of fluoride ingested and the stage of formation of the teeth at the time. Gedalia and Yardeni [49] found much more fluoride in the teeth when the water supply contained 1.3 ppm fluoride than when it contained 0.35 ppm. McClure and Likins [75] have shown that the teeth of individuals continuously exposed to an optimal amount of fluoride contained a higher percentage of fluoride than those exposed to water containing only 0.3 ppm fluoride. The enamel and dentine of the latter contained 0.0133 and 0.0385 per cent respectively compared with 0.0100 and 0.0240 per cent in the former. Exposure to high concentrations (2.5 - 5.0 ppm) was reflected in the fluoride results for dentine (.076) and enamel (.0345) per cent.

In general, fluoride concentrations are higher in dentine than enamel [35 56 75 89 94] and higher in the outer enamel than the inner [12 35]. Hargreaves and Weatherell [56] found that while the concentration of fluoride in deciduous enamel increased up to the age of four years and then remained constant, the concentration in dentine rose to its peak then showed a marked fall prior to exfoliation. The pattern was the same for both high and low fluoride areas.

35

Ericsson and Ullberg [35] demonstrated autoradiographically the accumulation of fluoride in the outer enamel and the inner dentine of developing teeth of rats. No uptake was visible in the erupted teeth.

Surface deposition of course occurs after eruption if exposure continues [40].

The foregoing information shows how fluoride is absorbed and made available for placental transfer. Its transfer to the foetal circulation and uptake by the foetus is not so clearly understood.

The magnitude of total transfer of a substance depends on the area of the surface across which transfer occurs, the rate of blood flow past the surface, the nature of the tissues separating the circulations and the chemical state of the substances being transported.

Some knowledge of placental anatomy is necessary to interpret any metabolic data.

The Placenta

The etymological meaning of the word 'placenta' is 'a flat cake'. This refers specifically to the human placenta. The animal placenta was defined by Mossman [80] as 'an intimate fusion of the foetal membranes to the maternal (or paternal) tissues for physiological exchange'.

The placenta usually referred to is a more restricted term equivalent to the chorio-allantoic placenta such as is found in all Eutherian mammals.

Functionally the placenta designates the region across which exchange of gaseous, nutritive and excretory products takes place between the foetal and maternal tissues, and especially between their respective blood streams.

Histologically the placenta constitutes those regions where foetal and maternal tissues are apposed, in such a way that the two blood streams are brought into close proximity.

There are three essentially different types of placenta in mammals, the chorio-vitelline, the chorio-allantoic and the chorionic [1].

The chorio-allantoic is the type present in higher mammals.

Grosser's Classification [54]

Four principal types of chorio-allantoic placenta have traditionally been recognised, according to the degree of erosion of the maternal tissue.

(a) Epitheliochorial: - a simple apposition of uterine epithelium and foetal chorion. The two blood streams are separated by maternal endothelium, connective tissue and epithelium and foetal trophoblast, connective tissue and endothelium.

(b) Syndesmochorial - erosion of maternal epithelium has taken place and there are now only five layers of tissue between the blood streams.

(c) Endotheliochorial - There has been erosion of maternal epithelium and connective tissue. Maternal and foetal blood are separated by the endothelium of the maternal blood vessels and the three layers of foetal tissues.

(d) Haemochorial - complete destruction of maternal tissues has occurred so that the maternal blood is directly in contact with foetal chorion.

Subsequently Mossman [79] suggested adding a fifth type, the haemo-endothelial placenta, to the types described by Grosser to accommodate the rabbit, rat and guinea-pig.

However, Amoroso [1] and Wislocki [113] have shown that the placental membrane of the rabbit and rat are also haemochorial.

TABLE I

TISSUES SEPARATING MATERNAL AND FOETAL BLOOD *

CLASSIFICATION	MATERNAL			FOETAL			EXAMPLES
	Endothelium	C.T.	Epithelium	Trophoblast	C.T.	Endothelium	
Epithelio chorial	+	+	+	+	+	+	Pig Horse
Syndesmochorial	-	+	+	+	+	+	Sheep Cow Goat
Endotheliochorial	-	-	+	+	+	+	Cat Dog Ferret
Haemochorial	-	-	-	+	+	+	Man Monkey Bat Mice Insectivores Rabbit Rat

* Adapted from a table presented by Amoroso [1] in 1961 and using the classification of Grosser [54].

While Grosser's morphologic classification is of importance in providing a ready means of histological indentification of placental types, it has several serious shortcomings. His view that the fewer the layers of tissue between the maternal and foetal circulations the greater the speed of exchange, has not been substantiated.

It has also been pointed out by Amoroso and others [2] that the number of layers varies in the same species from week to week, even in corresponding portions of the same placenta.

No longer is the placenta regarded as a simple semipermeable membrane, but is known to be an extremely complex organ.

A new functional classification has been suggested by Page [84] accounting for both the qualitative and quantitative selectivity of transfer.

This scheme encompasses the nature and size of the materials transported, the relative rates of transfer, the actual mechanisms involved in the transfer and the equality and inequality of distribution of certain substances between maternal and foetal tissues.

Whereas red blood corpuscles do not normally cross the placenta simple substances of low molecular weight, such as sodium, can diffuse across the placenta in both directions provided that they are not destroyed during transport [84]. However, some such substances exist in the blood as complex organic molecules and others, such as iron, are attached to proteins so that for any placenta the relative rates of transfer depend on constants peculiar to the substance to be transported [84].

The use of unstable isotopes in experimental work has greatly facilitated the study of transplacental mechanisms.

It has been found that certain substances are present in higher concentrations in foetal plasma than in maternal and that the permeability of the placenta is not necessarily the same at all stages of gestation. Changes in the mass of the placenta also occur as well as in its vascularity. In the early stages of gestation the placenta weighs more than the foetus, but at about half way through pregnancy the weights become approximately equal and the ratio then remains constant [8].

It has been found that there is more calcium and phosphorus in foetal plasma than in maternal [63 112]. Logothetopoulos and Scott [70] have shown that in the guinea-pig, rabbit and rat, inorganic iodide is higher in the foetal than in the maternal plasma.

Flexner and Pohl [42] found that the foetal guinea-pig received across the placenta about fifty times as much sodium as was incorporated in the growing tissues.

Gellhorn and Flexner [50] showed that water crosses the placenta 150 - 500 times more rapidly than the net uptake by foetal guinea-pigs indicates. Plentl [86] demonstrated a similar turnover in humans, finding that the amniotic fluid is replaced every three hours.

Wilde et al [112] concluded that unlike sodium and water, inorganic phosphate reaches the foetus in an amount only equal to the total phosphorus retained in growth. They also showed that in guinea-pigs the transfer rate of inorganic phosphorus per unit weight of placenta increases ten times between the thirty-first day of pregnancy and full term (70 days). A similar increase in sodium transfer has been found [42]. The change in rate of sodium transfer per unit weight of foetus follows the foetal growth curve.

Placental Transfer of Fluoride

There is only a limited amount of information on placental transfer of fluoride. The majority of this work involves the analysis of foetal calcified tissue and only a few studies relate maternal and foetal blood fluoride levels and placental fluoride content. (See Tables II and III)

Fluoride in Maternal and Foetal Blood

Humans

Ericsson and Malmnas [34], Gedalia et al [46 48], Held [58 59], Ziegler [115] and Auermann et al [4] have provided the only simultaneous estimation of maternal and foetal blood fluoride in humans. These authors are not in agreement on the relationship between maternal and foetal blood content and because the studies are so different, evaluation and comparison of the results is difficult.

Ziegler [115] found a higher fluoride content in maternal blood (0.28 ppm) than in foetal blood (0.15 ppm) and umbilical cord blood (0.13 ppm) in five women who had drunk milk containing 1 ppm fluoride during pregnancy. The maternal blood value was greater than that of women who were not given the additional supplement. The increase was from 0.15 ppm to 0.28 ppm but the corresponding foetal blood showed only a slight increase from 0.13 ppm to 0.15 ppm. Held [58] suggested that fluoride might be more concentrated in foetal than maternal blood when he provided a supplement in tea to thirteen pregnant women. The maternal values ranged from 0.17 to 0.38 ppm and simultaneous foetal values from 0.24 to 0.32 ppm. The higher foetal values paralleled the higher maternal values and in nine of the thirteen women foetal values were higher than maternal.

In a later study [59] when a supplement of 1.5 to 2.5 ppm per day was given, the results were similar.

No values or details of analysis were given by Auermann et al [4] but at 1 ppm fluoride in the drinking water they found that fluoride levels in maternal and foetal blood were approximately equal.

Gedalia et al [46] found no significant difference between the fluoride values of 0.09 ppm in maternal blood and 0.1 ppm in foetal blood in an area where the water supply contained 0.55 ppm fluoride. Placental tissue contained 0.15 ppm fluoride. The samples were obtained from groups of 25 or more healthy pregnant women just prior to delivery, from cord blood at normal term delivery and from whole placental tissue. Analysis of 25 non-pregnant women in the same area revealed a significantly higher blood fluoride content of 0.18 ppm.

More recently Gedalia et al [48] have tried to relate maternal and foetal levels to fluoride in the drinking water in groups exposed to high (0.6 - 1.1 ppm) and low (0.06 - 0.15 ppm) levels of fluoride.

There are three interesting points arising from this study. The first is that there is no significant difference between the mean fluoride values of cord blood (0.165 and 0.175 ppm) at high and low intake levels.

The second point is that in both high and low intake groups the mean fluoride values of maternal blood (0.150 and 0.234) were not significantly different from placental tissue (0.121 and 0.228).

The third and most interesting fact is that, whereas in the low fluoride group the mean fluoride value of cord blood was significantly higher than placenta and higher than maternal blood, it was just the opposite in the high fluoride group even though all values had increased. The investigators concluded that the placenta is freely permeable at low intake but that when the intake is high, it plays a regulating role.

Ericsson and Malmnäs [34] used an entirely different approach. They injected radioactive fluoride intravenously into four women who were to have therapeutic abortions. The F^{18} content of the foetal blood varied from one fourth to one tenth of the corresponding maternal blood.

It should be remembered that as blood containing fluoride returns from the placenta to the foetus considerable dilution will take place so that cord blood values could be expected to be higher than those of foetal heart blood.

Animals

The only investigation giving absolute values is that of Brzezinski et al [14]. Pregnant rats were fed up to 104.5 ppm fluoride and the maternal blood fluoride rose from 0.09 ppm to 0.14 ppm. No figures for foetal blood were given.

Ericsson and Malmnäs [34] studied the transfer of F^{18} in rabbits. Transfer to the foetal blood was limited and showed only a slight increase over the period of investigation. In no case did the foetal concentration reach one third of the simultaneous maternal level. This study demonstrated that even sudden increases in maternal concentration could not produce any great rise in the content of the foetal blood.

A most interesting study has recently been conducted by Bawden et al [6]. F^{18} was injected intravenously into pregnant sheep and samples of maternal and foetal blood were taken with the lamb still in utero. Although this placenta is calssified as syndesmochorial, the results of the study were similar to those of Ericsson and Malmnäs

Maternal clearance was rapid and over a period of eighty minutes foetal levels were low. The highest foetal level was only 15 per cent of the lowest corresponding maternal value. A 2 ml. foetal blood sample contained only about 0.0005 per cent of the injected dose.

When F^{18} was injected into the foetus clearance from the foetal circulation was rapid. Activity in the maternal circulation six minutes after injection indicates that fluoride can be transferred across the placenta from the foetus to the mother if the concentration gradient exists in that direction.

Although experimental procedures differ and absolute values vary considerably, the evidence indicates that in humans and animals maternal blood contains a higher concentration of fluoride than foetal blood. It has also been suggested that at low intake the position is reversed. Experiments with F^{18} have demonstrated that maternal clearance is rapid, that the transfer can be a two-way process and that sudden increases in maternal fluoride do not produce corresponding increases in foetal fluoride.

Fluoride in the Placenta and its Relation to Blood Fluoride

The amount of fluoride in placental tissue has been expressed in ppm measured on the basis of weight or volume. Only where blood values have been similarly expressed can the two be compared.

Humans

All investigators have found greater concentrations of fluoride in the human placental tissue when a supplement is administered than when the intake of fluoride is dependent on the traces normally present in the diet [38 39 40 43 46 48 115], but absolute values obtained vary considerably. (See Table II)

Gardner et al [43] found 2.09 ppm fluoride in the placenta when there was a supplement of about 1 ppm. When the water supply contained only 0.06 ppm fluoride, the placenta contained 0.74 ppm which is nevertheless considerably more than the value

of 0.228 ppm found by Gedalia et al [48] when approximately 1 ppm of fluoride was ingested during pregnancy.

Values obtained by others are intermediate between those of Gardner and Gedalia.

Ziegler [115] found 0.684 ppm fluoride in placental tissue of women who drank milk containing 1 ppm fluoride and 0.362 ppm in controls.

When a fluoride supplement of 1.2, 1.0 or 0.825 mg/day was given in tablet form, Feltman [38], and Feltman and Kosel [39 40] found placental fluoride values ranging from 0.85 ppm to 1.49 ppm. There was not a marked difference between these values and controls and it was found on analysis that certain pharmaceuticals being taken as prenatal medication contained fluoride [40]. No data is available on the fluoride content of the placenta at ingestion levels greater than 1.2 ppm, but Ericsson and Malmnas [34] found no accumulation in the placenta after injection of F^{18} into pregnant women. Measuring all activities on a weight basis, the F^{18} content of the placental tissue was between that of maternal and foetal blood.

Other investigators, Ziegler [115], Gardner et al [43], and Gedalia et al [46] have found placental values higher than maternal blood but except for those of Gedalia it is doubtful whether a comparison can validly be made. Gedalia et al [46] found, in all cases where the water supply contained 0.55 ppm, there was significantly more fluoride in placental tissue than in maternal blood, using 15 ml. as the sample for analysis but there was no positive correlation between the two. In a later study Gedalia et al [48] found that the mean fluoride values of placental tissue and maternal blood were not significantly different for either low (0.15 ppm) or high (1.1 ppm) intake of fluoride.

Relating mean fluoride values of cord blood and placental tissues, Gedalia et al [46 48] found that when the fluoride intake was 0.06 - 0.15 ppm the mean fluoride value of cord blood was significantly higher than that of placental tissue, but when the intake was 0.55 or 0.6 to 1.0 ppm the placental content was significantly higher than the cord blood content, with a positive correlation existing.

Ziegler [115], Feltman [38] and Feltman et al [39 40] have also reported considerably higher

fluoride content in placental tissue than cord blood when the ingested fluoride ranged from 0.825 to 1.2 mg per day.

Feltman et al [39 40] analysed whole placentas and found that the fluoride was concentrated in the periphery. They suggested two possible explanations for this, one being that the high peripheral fluoride could be related to the high calcium content at the periphery. The other suggestion was that, in an attempt to prevent excess fluoride reaching the foetus, the placenta might push it away from the area of most active exchange. The former idea seems to be the more reasonable.

Animals

Fluoride as F^{18} has been detected in placental tissue in rats [23 35], rabbits [34], mice [33 35], and cattle [9]. Only for cattle are there any quantitative data available. Bell et al [9] found that when cows had been fed up to 57 ppm fluoride in the diet for eight years the maternal and foetal placentas contained 0.50 and 0.23 ppm respectively. The content was not related to the quantity ingested. When F^{18} was injected the activity was higher in the maternal placenta than in the foetal. Feeding of stable fluoride had no effect on the metabolism of F^{18}.

Cohen et al [23] reported measurable amounts of fluoride in the placenta after intraperitoneal injection of F^{18} into rats in the second and third trimesters of pregnancy.

Ericsson and Malmnäs [34] found the level of fluoride in the rabbit placenta to lie between that in maternal and foetal blood. They did not detect any accumulation in the placenta after a single intravenous injection of F^{18}. In autoradiographic investigations Ericsson and Ullberg [35] demonstrated the relation of the localisation of F^{18} in the placenta to the increase of macroscopic and microscopic precipitates of calcium salts.

Ericsson and Hammarström [33] confirmed this relationship when they injected Ca^{45} and F^{18} into pregnant mice. Autoradiographs made three and four minutes after injection showed that the concentration of fluoride in the placenta was about the same as in the maternal blood. After fifteen minutes little F^{18} remained in the

blood or placenta but where areas and spots did occur they were found to coincide with areas that had taken up Ca^{45}.

F^{18} was not found localised in the foetal skeleton but appeared to pass from the placenta back to the mother. Ericsson and Hammarström[11]'s work suggests that the low fluoride content of foetal blood compared to maternal blood may be due, not to rapid foetal clearance because little F^{18} was found in the foetal skeleton, but to a combination of slow placental diffusion and efficient homeostatic mechanism of themammalian body.

A variety of figures has been given for the amount of fluoride in placental tissue in man and animals. There is always more fluoride present when water is fluoridated or a supplement is given than when only normal dietary fluoride is available. However, the data obtained on cattle [9], (values of the same order of magnitude as in humans), indicate that marked increases in the amount ingested do not alter the content. In addition studies with radiofluoride indicate that there is little or no accumulation in the placenta,either in man or animals. Areas of calcification are, however, potential sites for fluoride accumulation. The relationship of placental tissue content to maternal blood content is not quite clear. According to some authors the placenta has a higher fluoride concentration in ppm than maternal blood but Gedalia et al [48] and Ericsson and Malmnäs [34] have found similar or even lower amounts in the placenta. It has been generally found that placental fluoride is greater than foetal blood fluoride in all species except at very low intake. A slow diffusion rate and rapid maternal homeostasis has been suggested as a possible explanation.

Fluoride in the Foetus

Humans

Little data is available concerning the fluoride content of foetal bones and teeth. (Table IV).

Martin [72] found average values of 20 ppm and 19 ppm fluoride in femurs, and maxilla and mandible, on a dry fat free basis, in nine months old foetuses where the

water supply content was about 1 ppm fluoride.

Yudkin [114] and Gedalia et al [47] reported increased amounts of fluoride in these bones when fluoride intake increased. Yudkin reported a sevenfold increase when the fluoride content of the water supply rose from 0.1 to 1.0 ppm.

Gedalia et al [47] found that the femur and mandible fluoride content in an area where the water supply contained 0.5 ppm fluoride was significantly greater than the content of these bones where the water contained 0.1 ppm fluoride and this difference increased with increasing foetal age. It was also found that the content of these bones increased considerably with foetal age when the drinking water contained 0.5 ppm or more [13 47] but that there was not an appreciable increase when the water contained only 0.1 ppm fluoride.

Gedalia et al [47] also found that the fluoride content of the teeth increased with advancing age in the 0.5 ppm groups but not in the 0.1 ppm group. There was more fluoride in the teeth of the 0.5 group than the 0.1 group at all foetal ages.

Martin [72] found an average of 12.5 ppm fluoride in foetal tooth buds at term when the water supply contained 1.0 ppm. Yudkin et al [114] claimed a fivefold increase in tooth bud content with an increase in the water content from 0.1 to 1.0 ppm fluoride.

Blayney and Hill [11] report the analysis of foetal tooth buds by Yudkin et al [114]. In a fluoridated area toothbuds of a thirty-six weeks foetus contained 89.2 ppm fluoride and a thirty-five weeks foetus 45.8 ppm. Foetal tooth buds at 44 weeks contained 10.3 ppm where there was only a trace of fluoride in the water supply.

Animals

Data is available indicating that there is an uptake of fluoride in foetal bones and teeth of rats [14 15 16 23 36 68 71 82 101], mice [33 35 41], rabbits [71], dogs [65], cattle [9 37 53 102], sheep [6 7] and guinea-pigs [61]. (See Table V).

It has generally been accepted that, in rats, at least 10 ppm fluoride is required in the maternal diet before there is any considerable uptake by the foetal skeleton

14 15 36 68 71 82, although some fluoride has been found in the foetus even at low levels [15 36]. When the supplement is increased the amount of storage in the foetus increases but not necessarily in proportion,

Buttner and Muhler [15] found that a twenty to fiftyfold increase in the fluoride content of drinking water of pregnant rats resulted in only a two or threefold fluoride increase in the carcass.

Maplesden et al [71] gave pregnant rats supplements of 0, 50, 100 and 200 ppm fluoride and found 0, 1.0, 1.8 and 4.1 ppm in the foetus on a wet basis.

When rabbits were fed the same amounts the foetus contained 3.1, 5.8, 9.3 and 15.8 ppm respectively.

With dietary supplements up to 14.5 ppm fluoride Brzezinski et al [14] obtained slight increases in the foetal bone content up to a mean content of 0.487 ppm. With 54.55 and 104.55 ppm the ashed foetal bones contained 3.0 and 3.8 ppm respectively. Ashed femurs of young rats whose mothers drank water containing 25 ppm fluoride during pregnancy were found by Stookey et al [101] to contain 161 ppm fluoride compared with 149 ppm in controls. This 12 ppm seems high compared with the results of other investigators, but may be a result of providing the supplement in the drinking water rather than in the diet.

Very little F^{18} is seen in the foetal rat skeleton in autoradiographs prepared after a single intravenous injection in rats and mice [33 35].

Hudson and Stookey [61] found indications that as little as 1.0 ppm fluoride in the drinking water passes the guinea-pig placenta and increasing concentrations up to 50 ppm result in increased foetal concentration.

In cattle Evans et al [37] found an average of 37 ppm of fluoride in foetal bone ash with unknown variable intake and Bell [9] detected F^{18} in six month foetal metatarsals in greater quantity than corresponding mature tissue. As much as 730 ppm was reported in foetal dog carcasses after dogs had ingested 5 - 25 ppm during pregnancy and 3250 ppm when a large dose of 50 mg was administered over 7.5 weeks [65].

Bawden et al [7] calculated that after two hours the total amount of F^{18} in two

foetal lambs was 0.16 and 0.42 per cent of the injected dose.

These authors had previously found that quantities per gm in foetal teeth and bone were approximately equal [6].

Only two other investigators have mentioned fluoride uptake in foetal teeth.

Buttner and Muhler [16] concluded that administration of 50 ppm fluoride per day to pregnant rats during the entire pregnancy produced a significant reduction in solubility of the teeth of the offspring.

Fleming and Greenfield [41] noted alteration in ameloblasts and retardation of enamel matrix formation in new born mice when the mother had ingested 600 - 1000 micrograms of fluoride.

In humans the fluoride in foetal bones and teeth increases with increased intake by the mother, but the increase is not particularly marked. At medium intake (0.5 ppm) the content increases significantly with foetal age but this does not occur at low intake (0.1 ppm).

In animals only slight transfer takes place below about 10 ppm in the diet or drinking water. Above this level the content of bones increases as the intake increases, although not in direct proportion. As expected, the fluoride reaching the foetus is concentrated in the bones and teeth, probably in similar concentrations.

TABLE II

SUMMARY OF RESULTS OF STUDIES OF FLUORIDE CONTENT OF MATERNAL BLOOD, PLACENTAL TISSUE AND FOETAL BLOOD OF HUMANS

AUTHOR	SUPPLEMENT ppm	METHOD	MATERNAL BLOOD ppm	PLACENTA ppm	FOETAL BLOOD ppm
Gardner et al [43]	0.06	water	-	0.74	-
	1.0 - 1.2	supply	-	2.09	-
Ziegler [115]	control	-	0.151	0.362	0.133
	1.0	fluoridated milk	0.278	0.684	0.153
Feltman and Kosel [39]	1.1 - 1.2	CaF_2 or NaF tab.	-	1.11	0.41
	control	-	-	1.01	0.17
	1.0	water supply	-	0.85	0.38
	control	-	-	0.67	0.22
Feltman [38]	0.825	tab.	-	0.90	0.22
Feltman and Kosel [40]	0.85	Na_2PO_3F	-	1.07	0.269
	1.0	CaF_2	-	1.09	0.448
	1.2	NaF	-	1.49	0.327
	control	Fluoride in medications	-	1.05	0.129
Gedalia et al [46]	0.55	water supply	0.09	0.15	0.11
Gedalia et al [45]	0.5 - 0.6	water supply	0.150	0.21	0.165
	0.9 - 1.1	" + tab.	0.234	0.228	0.175
Ericsson and Malmnäs [34]	F^{18}	intravenous injection	M >	P no accumulation >	F

TABLE III

SUMMARY OF RESULTS OF STUDIES OF FLUORIDE CONTENT OF MATERNAL BLOOD, PLACENTAL TISSUE AND FOETAL BLOOD OF EXPERIMENTAL ANIMALS

AUTHOR	SUPPLEMENT	METHOD	MATERNAL BLOOD	PLACENTA	FOETAL BLOOD
Bnzezinski et al [14] Rat	0.2 ppm	normal diet	0.1 ppm	-	-
	5.2 ppm	(4.55 ppm food (0.65 ppm water	0.09 ppm	-	-
	104.55 ppm	(4.55 ppm food ((100 ppm water	0.14 ppm	-	-
Ericsson and Malmnäs [34] Rabbit	F^{18} 8-10 μCi per Kg	intravenous injection	Maternal	always least ratio 3/1	> Foetal
Bawden et al [6] Sheep	F^{18}	intravenous injection	Maternal	always least ratio 8/1	> Foetal
Bell et al [9] Cattle	F^{18} 400 μCi 47 ppm for 8 years	injection in diet	Maternal	> ratio 6/2 Maternal placenta Foetal placenta	Foetal 0.50 ppm 0.23 ppm
Cohen et al [23] Rat	F^{18}	intraperitoneal injection	present	present	-
Ericsson and Ullberg [18] Rat and Mouse	F^{18}	intravenous injection	-	present in relation to calcification	-
Ericsson and Hammarström [33] Mouse	F^{18} 1-5 μCi per gm	intravenous injection	-	present in relation to calcification	-

TABLE IV

SUMMARY OF RESULTS OF STUDIES OF FLUORIDE CONTENT OF BONES AND TEETH OF HUMAN FOETUSES

AUTHOR	SUPPLEMENT ppm	FOETAL AGE	FEMUR ppm	MANDIBLE (+ MAXILLA) ppm	TOOTH BUDS ppm
Martin [72] (dry fat free sample)	1.0 water supply	9 months	20	19	12.5
Yudkin et al [114]	increased from 0.1 to 1.0 water supply	9 months	increase sevenfold	increase sevenfold	increase fivefold
Blayney and Hill [11]	1.0	36 weeks	-	-	89.2
	"	35 "	-	-	45.8
	trace water supply	44 "	-	-	10.3
Gedalia et al [47] (ashed sample)	0.05 to 0.1 water supply	5 months	35.7	32.7	-
		6 "	39.6	42.3	30.9
		7 "	40.7	39.0	34.0
		8 "	42.3	38.5	31.7
		9 "	43.8	46.9	40.8
	0.5 to 0.6 water supply	4 months	20.8	23.8	-
		5 "	56.9	31.7	26.3
		6 "	59.0	47.0	32.6
		7 "	71.6	53.5	43.0
		8 "	79.4	66.0	57.9
		9 "	92.5	78.8	69.7
Brzezinski et al [13]	0.55 in water supply	3 months	2.5	-	-
		4 "	5.0	-	-
		5 "	32	-	-
		6 "	56	-	-
		7 "	77	-	-
		8 "	100	-	-
		9 "	130	-	-

TABLE V

SUMMARY OF STUDIES OF FLUORIDE CONTENT OF FOETAL BONES AND TEETH IN EXPERIMENTAL ANIMALS

AUTHOR	SUPPLEMENT	METHOD	FOETUS		TEETH
			site	ppm	ppm
		DOG			
Knouff el at [65]	5 ppm for 8 weeks	drinking water	whole foetus	0 *	-
	25 ppm through gestation	"	whole foetus	730 *	-
	50 mg. over 7.5 weeks	diet	whole foetus (2 weeks prem.)	3250 *	-
		RAT			
Brzezinski et al [14]	Throughout gestation				
	0.2 ppm	diet trace	skeleton	.317 ✕	-
	5.2	" + 5 ppm water	skeleton	.339 ✕	-
	5.2	4.55 diet + 0.65 water	"	.318 ✕	-
	14.55	0.55 diet + 10.0 water	"	.487 ✕	
	54.55	4.55 diet + 50 water	"	3.062 ✕	-
	104.55	4.55 diet + 100 water	"	3.833 ✕	-
Buttner and Muhler [15]	Throughout gestation				
	control	drinking water	carcass of one day old rat	53 ✕	-
	1 ppm	"		50 ✕	-
	3	"		51 ✕	-
	5	"		45 ✕	-
	10	"		77 ✕	-
	25	"		81 ✕	-
	50	"		115 ✕	-

TABLE V (Cont'd.)

AUTHOR	SUPPLEMENT	METHOD	FOETUS		TEETH
			site	ppm	ppm
		RAT			
Buttner and Muhler [16]	control	drinking water		-	control
	50 ppm through pregnancy	"			reduced solubility
	50 ppm through pregnancy and lactation	"			"
Cohen et al [23]	F^{18}	intraperitoneal injection	whole foetus	detectable 1-2 hours	- -
Lehman and Muhler [68]	0.5 ppm 25 " 50 "	as NaF in drinking water	foetus	increase proportional	- - -
Maplesden et al [71]	a. 10 ppm 50 100 200	added as NaF to basal diet	whole foetus	0.0 ∅ 1.0 ∅ 1.8 ∅ 4.1 ∅	- - - -
		3 ppm F			
	b. 0 ppm 50 100 200 300	throughout gestation " " "	first litter whole foetus	0.3 ∅ 1.4 ∅ 1.7 ∅ 2.8 ∅ 5.6 ∅	- - - - -
	c. 0 ppm 50 100 200 300	throughout gestation " " "	second litter whole foetus	1.0 ∅ 0.0 ∅ 2.0 ∅ 1.8 ∅ 3.5 ∅	- - - - -
	d. 0 ppm 50 100 200	throughout gestation " "	third litter whole foetus	0.2 ∅ 0.0 ∅ 0.3 ∅ 6.9 ∅	- - - -
	e. 0 ppm 50 300	throughout gestation "	whole foetus	0.0 ∅ 0.6 ∅ 3.5 ∅	- - -
Murray [82]	control	-	whole foetus	1.1 ∅	-
	225 ppm	NaF added to diet		5.5 ∅	-

TABLE V (Cont'd.)

AUTHOR	SUPPLEMENT	METHOD	FOETUS		TEETH
			site	ppm	ppm
		RAT			
Stookey et al [101]	control	-	femur	149	-
	25 ppm	drinking water	"	161	-
		MOUSE			
Ericsson and Hammarström [33]	F^{18}	Intravenous injection	skeleton autoradiograph	Low in relation to maternal	-
Ericsson and Ullberg [35]	F^{18}	Intravenous injection	skeleton autoradiograph	present after 30 minutes	-
Fleming and Grenfield [41]	60 - 80 µg	drinking water	skull	retarded calcification	changes in ameloblasts
	600 -1000 µg	"	"		
		RABBIT			
Maplesden et al [71]	0 ppm	added as NaF to basal diet	whole foetus	3.1 ◊	-
	50		"	5.8 ◊	-
	100		"	0.3 ◊	-
	200	46 ppm throughout gestation	"	14.8 ◊	-
	300		"	21.5 ◊	-
		COW			
Bell et al [9]	F^{18}	injected	metatarsal	present	-
Evans et al [37]	variable	unspecified	embryonic bone	37 ℵ	-
		"	foetal bone	30 ℵ	-
Greenwood et al [53]	10 - 109 ppm	diet throughout	bone	correlated with intake	-
Suttie et al [102]	control	diet	metatarsal	11 *	-
	50 ppm for 5 years	"	"	140 *	-

TABLE V (Cont'd.)

AUTHOR	SUPPLEMENT	METHOD	FOETUS		TEETH
			site	ppm	ppm
		SHEEP			
Bawden et al [6]	F^{18}	intravenous	bone	700-1000 ◊ cpm/mg	800-1500 ◊ cpm/mg
Bawden et al [7]	F^{18}	intravenous	bone + carcass in 2 hours	0.16% of dose 0.42% of dose	- -
		GUINEA-PIG			
Hudson and Stookey [61]	1-50 ppm throughout gestation	drinking water	foetus	present at all concent-rations	-

* Dry fat free

◊ Wet basis

Ⅹ Ash

EXPERIMENTAL INVESTIGATION

I. MATERIALS

a. Radioisotope

The only satisfactory isotope of fluorine for tracer work is F^{18}, which has a half-life of 112 minutes and decays to O^{18} with the emission of a positron, resulting in the formation of two gamma (γ) rays, each with an energy of 0.51 Mev travelling in opposite directions.

The limited half-life of this isotope restricts its use to short term investigations unless large quantities are produced. However the rapid decay obviates the necessity for disposal or storage of radioactive waste.

In this study F^{18} was supplied carrier free in a sterile solution of sodium chloride with a molarity from 0.08 to 1.0 and a pH between 7.0 and 8.0. It was produced at the Australian Atomic Energy Research Establishment, Lucas Heights, by neutron irradiation of lithium carbonate for six hours at flux of 9×10^{12} $n/cm^{-2}/sec^{-1}$.

A volume of 1.0 to 2.0 ml of solution was used in each experiment so that the total calculated activity at the time of injection varied from 0.2 to 0.5 millicuries (MCi). The injected dose was never more than 2.6 microcuries (μCi) per gram of body weight.

b. Animals

One non-pregnant female rat and sixteen pregnant white rats of either Sprague-Dawley or Wistar strain were used. Each animal was weighed prior to the experiment. The total rat plus litter weight ranged from 300 to 500 grams.

Experiments were carried out at varying stages of gestation from seventeen to twenty-one days. At less than seventeen days the foetuses were fragile and easily damaged by handling. Their small size also made blood sampling extremely difficult. Near term the placenta tends to bleed profusely and can be accidentally damaged readily.

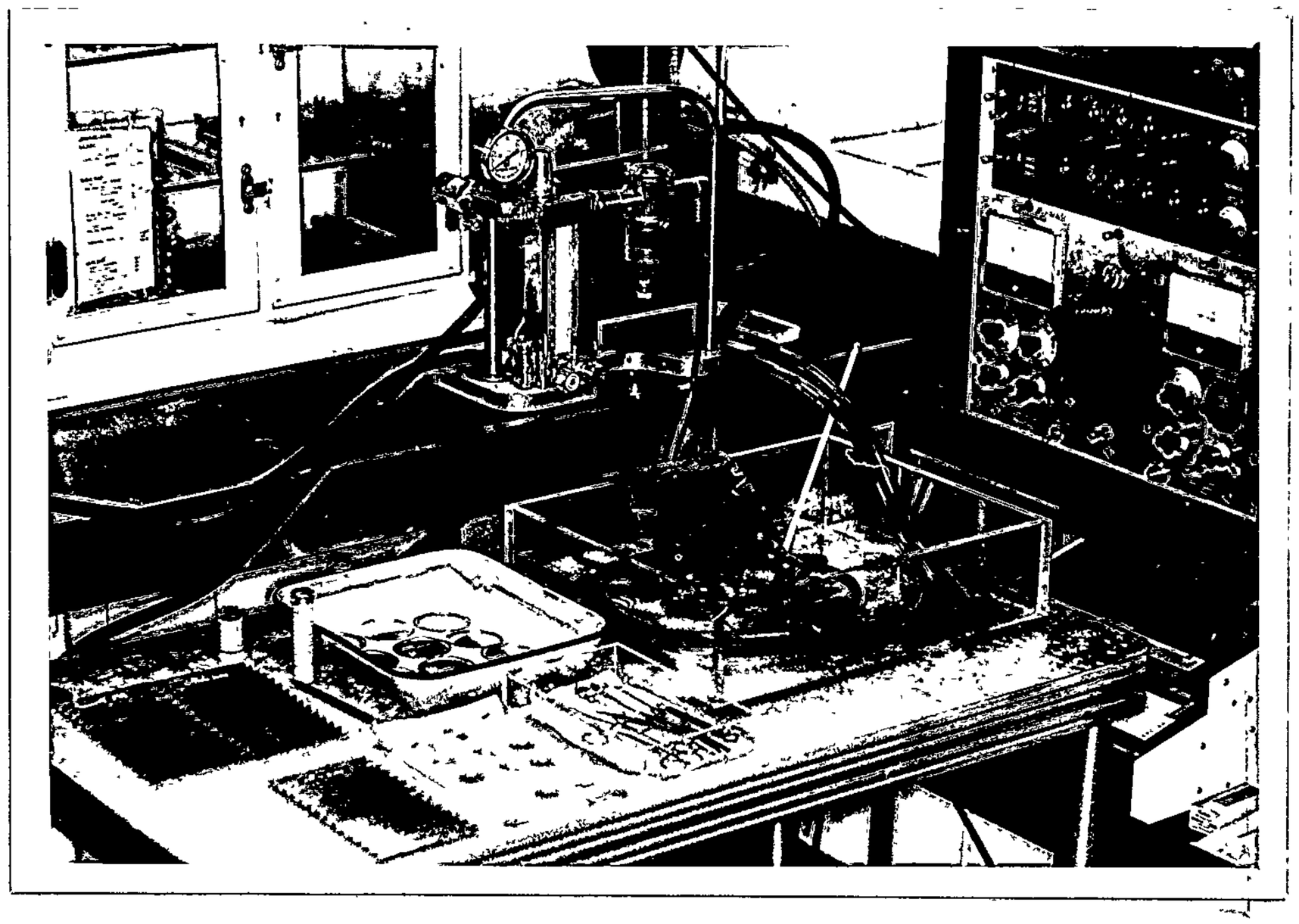
c. Equipment

Figure I shows the apparatus set up ready for the commencement of an experiment.

Details of the radiation detecting equipment can be found in Appendix I.

FIGURE I

EXPERIMENTAL APPARATUS



Experimental Apparatus shown:
- Constant temperature bath
- Supporting platform in bath
- Anaesthetic supply - N_2O, O_2, Ether
- Surgical instruments
- Planchettes; large - foetus
  small - placenta
- Trays for blood sample pipettes;
  maternal - foetal - placental
- Radiation scaler

2. SURGICAL PROCEDURE

The technique used is a modification of that described by Reade and Jenkin[88].

a. Anaesthesia

After induction with ether the trachea was exposed by lifting aside the sternohyoid and sternothyroid muscles and an aneurysm needle passed beneath it. Taking care to maintain a dry field a small horizontal incision was made on the anterior surface of the trachea (Figure II) and a stainless steel cannula inserted. This was tied securely with suture silk and connected to the anaesthetic gas supply (Figure III). The animal was then maintained on a mixture of nitrous-oxide, oxygen and ether at a rate of about 200 ml per minute for as long as required.

b. Preparation of the Injection Site

The femoral vein was selected as a suitably large and easily accessible vein for administration of the isotope solution.

It was desirable that the radioisotope be injected directly into the blood stream to avoid the time lag required for absorption so that foetal uptake could be related directly to maternal clearance.

The site selected was on the medial aspect of the hind leg where the femoral vein is readily visible, superficial and medial to the artery.

FIGURE II

TRACHEAL INCISION

Fig. II shows Trachea exposed
aneurysm needle passed beneath trachea
incision on anterior wall of trachea

FIGURE III

TRACHEAL CANNULA INSERTED

c. Foetal Externalization

The mother rat was placed on the specially made sloping platform in a bath of Ringer-Locke solution maintained at 37 degrees Centigrade, the solution reaching the base of the thorax to cover the foetuses without exerting undue pressure on the thorax of the mother.

After removing a flap over the lower part of the abdomen, the bicornuate uterus was exposed by splitting the abdominal muscles. The uterus was then externalized and an antemesometrial incision made exposing the embryonic sacs.

The foetal membranes (yolk sac and amnion) were cut, leaving each foetus exposed and attached by its umbilical cord to the placenta. (Figure IV). Exposure of the foetuses before injection of the radioisotope ensured that purely placental transfer was measured. In two cases however several foetuses were left in their sacs so that measurement of the F^{18} content of the amniotic fluid could be carried out.

d. Administration of the Radioisotope

The needle was inserted into the femoral vein at the point of junction with the great saphenous vein. The time at which the injection was commenced and completed was noted and the time of commencement was called T_o. The whole injection time was usually from twenty to thirty seconds.

FIGURE IV

FOETAL EXTERNALIZATION

Fig. IV shows Anaesthetised rat in bath foetuses and placentas exposed

3. SAMPLING

a. Series of Foetuses and Placentas

Foetuses and their corresponding placentas were removed serially from the uterus over periods extending up to two and a half hours and the exact time of removal noted. They were dried with absorbent paper, separated and placed on individual aluminium planchettes ready for measurement of their radioactive content.

b. Blood Samples.

Capillary pipettes with a volume of 0.02 ml were used to obtain all blood samples. These tubes were of consistent bore and standard length so that if a tube was not completely filled the volume of the sample could be calculated proportionally.

i. Maternal Blood

Blood was obtained from the tail, the first sample as soon as possible after the injection was completed and succeeding samples at times nearly corresponding to the removal of the foetuses.

ii. Foetal Blood

Blood was obtained directly from the heart of the foetus, after its removal from the mother, through an opening made into the thoracic cavity. In two studies the samples were obtained from the foetus at the point of severence of the umbilical cord.

iii. Placental Blood

A sample of blood was obtained from the placenta by making a radial incision in the circumferential region (Figure V). This sample was considered to be mixed maternal and foetal blood.

4. METHOD OF F^{18} ANALYSIS

a. Whole Foetus and Whole Placenta

The total activity of the placentas and foetuses was measured with a shielded 1" x 1" scintillation crystal. (Figure VI). Constant geometry was maintained throughout the experiments and the efficiency of the system was calculated by using a standard source. The activity of the samples was such that thirty-second counts were long

enough to be sufficiently accurate. Background counts were obtained at intervals throughout the counting procedure.

In addition to duplicate counts in each experiment, a series of multiple readings for one foetus and placenta was carried out.

The exact time of counting each sample was noted.

b. Blood Samples

The tubes containing blood samples were broken into short lengths and counted with a scintillation counter for sixty seconds each. The exact time at which the sample was counted was noted. Background was checked at regular intervals.

The efficiency of the system was calculated using a standard source.

For details of counting equipment see Appendix I.

FIGURE V

OBTAINING PLACENTAL BLOOD SAMPLE

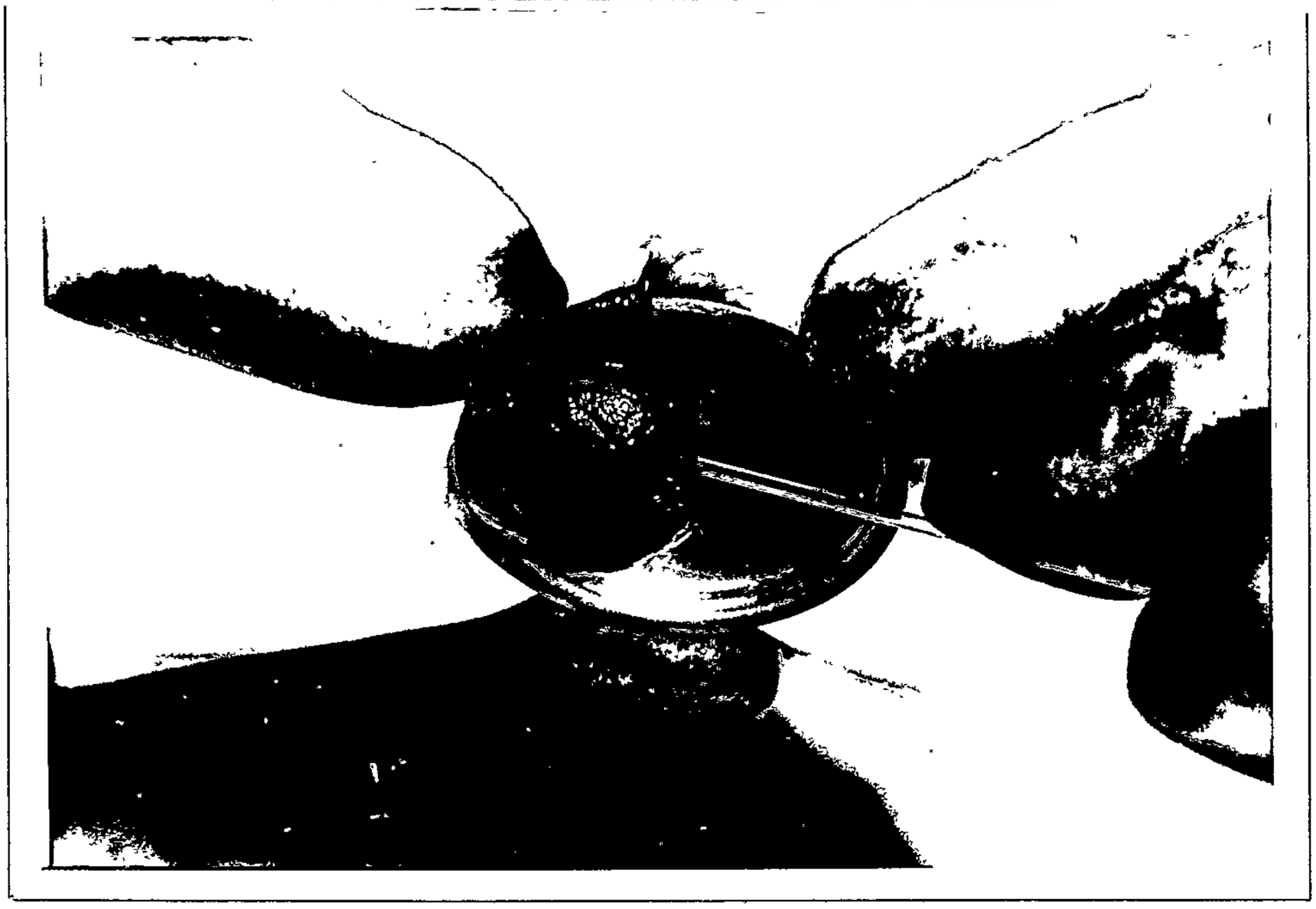


FIGURE VI

COUNTING SYSTEM FOR WHOLE FOETUS AND PLACENTA

5. RECORDING OF DATA AND CALCULATION OF RESULTS

A sample of the data tabulated at the commencement of each experiment can be seen in Figure VII.

The following additional data was recorded for each experiment:

1. The time the sample was taken.
2. The time the sample was counted.
3. The total number of counts and the length of counting time.
4. The background count at intervals throughout the experiment.
5. The sample size. This applied to blood samples only when the standard guage 0.02 ml tube was not completely filled.

To calculate the results all counts were first corrected for background.

In the case of the blood samples correction to a volume of 0.02 ml was next made so that results could readily be compared.

All values were corrected for decay to T_o using Table VI. These corrected values were then expressed as a percentage of the administered dose, taking into account the efficiency of the counting system. The mean of the two sets of values was then calculated, giving the final value.

FIGURE VII

TYPICAL GENERAL DATA SHEET

PRENATAL TRANSFER STUDY Date 12·10·66
E. Bendeich

Experimental Animals

Weight of Rat (grams) (including foetuses)	495
Number of foetuses	10
Age of foetuses (days)	19 - 20
Crown rump length (mm)	38

Radioisotope Supplied

F^{18}

Preparation	Neutron irradiation $Li(O_3$ 6 hrs. low flux
Solution	0·15 M NaCl pH 7
Volume (ml)	1·5
Activity	0·5 MCi
Time	11·00 a.m.

Dose at T_o

Volume (ml.)	1·5
Activity	0·20 MCi - 0·4 μCi/gm
Time Commenced Injection (T_o)	1.28 p.m.

TABLE VI

DECAY OF F18

Time in minutes.

Minutes	0	1	2	3	4	5	6	7	8	9
0	1;00	.994	.987	.981	.976	.970	.963	.957	.952	.946
10	.940	.934	.929	.923	.917	.912	.906	.900	.895	.889
20	.883	.878	.873	.868	.863	.857	.852	.846	.841	.836
30	.831	.826	.820	.815	.810	.805	.800	.796	.791	.786
40	.781	.776	.771	.766	.761	.756	.752	.747	.743	.739
50	.734	.729	.725	.720	.716	.712	.707	.703	.699	.694
60	.690	.686	.682	.677	.673	.669	.664	.660	.657	.653
70	.649	.644	.640	.636	.633	.629	.625	.621	.617	.613
80	.609	.606	.602	.599	.595	.591	.587	.583	.580	.576
90	.572	.569	.566	.563	.559	.556	.552	.549	.545	.542
100	.539	.535	.532	.529	.526	.523	.520	.516	.513	.510
110	.507	.503	.500							

Calculation of Percentage Dose Recovery:

Dose = MCi of activity in injected solution at T_o

1 MCi = 3.7×10^7 counts/second

Count = Count recorded corrected to T_o

Time = Length of time counted (seconds)

Efficiency Factor = Efficiency of counting systems used
For whole foetus and placenta = 1%
For blood samples = 10%

$$\frac{\text{True Counts/second}}{(\text{at } T_o)} = \frac{\text{Count}}{\text{Time}} \times \text{Efficiency Factor}$$

$$\text{Percentage Dose Recovery} = \frac{\text{True Count/sec}}{\text{Dose} \times 3.7 \times 10^7} \times \frac{100}{1}$$

Figures VIII and IX show a typical set of calculations of percentage dose recovery for whole foetus and for foetal blood samples.

The calculated results were finally expressed graphically on a semi logarithmic scale for percentage dose recovery against time.

FIGURE VIII

TYPICAL WORK SHEET - WHOLE FOETUS

EXPERIMENTAL DATA

E. Bendeich

Date : 12·10·66

Study Series of Foetuses Removed (whole count)

MATERNAL DOSE (MCi) COUNTING EFFICIENCY BACKGROUND COUNT

0·20 MCi = 0·4 μCi/gm 10% (N·618A) Variable B.G.

Sample Taken Time *	Time Counted	Number of Counts	Counts Corrected Background		Sample Size	Counts Corrected Size	Counts Corrected to T_o	Dose Recovery
min sec	min sec	/30sec	B.G.		gm / sec	/ sec	/ sec	per cent
24	32	302	125	177	4·612		216	·00973
24	51	266	136	130	"		178	·00802
							·00887 #	
1	35	1409	137	1272	5·073		1580	·07117
1	53	1282	136	1146	"		1592	·07171
							·07114 #	
2 30	37	694	133	561	4·852		705	·03176
2 30	57	578	140	438	"		623	·02806
							·02991 #	
4	40 30	648	130	518	4·881		665	·02995
4	65 30	623	116	507	"		760	·03423
							·03209 #	
6 30	42 30	794	142	652	4·685		848	·03820
6 30	68	633	116	517	"		787	·03545
							·03682 #	
20	46 30	644	118	526	2·433		701	·03158
20	70	548	93	455	"		701	·03158
							·03158 #	
60	63 30	1960	116	1844	4·829		2732	·12306
60	72	1921	93	1828	"		2856	·12865
							·12585 #	
90	131	1409	98	1311	4·652		2772	·12486
90	167	1071	87	984	2		2764	·12450
							·12468 #	

* All times from T_o injection commenced

\# Mean of two readings

FIGURE IX

TYPICAL WORK SHEET - FOETAL BLOOD

EXPERIMENTAL DATA

E. Bendeich

Date : 12·10·66

Study Foetal Blood Samples, 0·02 ml.
(from umbilical cord when severed)

MATERNAL DOSE (MCi) COUNTING EFFICIENCY BACKGROUND COUNT

0·20 MCi, 0·4 μCi/gm 10% (N:664A) 104/60 sec.

Sample Taken Time *	Time Counted	Number of Counts	Counts Corrected Background	Sample Size	Counts Corrected Size	Counts Corrected to T_o	Dose Recovery
min sec	min sec	/60 sec	104/60 sec	mm	/60 sec	/60 sec	per cent
24	106 30	725 #	673 #	37	2323	4485	·0202
1	108	180 #	128 #	33	497	969	·0044
2 30	109 30	363	259	61	272	504	·0011
4	111	332	228	35	417	829	·0019
6 30	113	282	178	60	189	381	·0008
20	114 30	222	118	13	581	1181	·0026
60	116	285	181	64	181	371	·0008
90	117 30	214	110	55	128	265	·0006
121	148	213	109	61	114	285	·0005

* All times from T_o injection commenced

counts/30 sec.

RESULTS

The results of the sixteen experiments summarized in Appendix II are presented here graphically as the percentage of a maternally administered dose of F^{18} recovered in the whole foetus and placenta and in 0.02 ml of maternal, placental and foetal blood.

1. WHOLE FOETUS

Each of the fifteen curves in figure X shows the amount of F^{18} in different members of a series of foetuses exposed to the radioisotope for different periods of time.

If the slight change in experimental conditions that occurs as each foetus is removed is disregarded then any curve may be said to indicate the accumulation of F^{18} in a single foetus. The percentage of the administered dose present at any one time can then be determined.

The same pattern of accumulation was found in all studies although there was some variation in the absolute values. Fluoride could be detected in the foetus within thirty seconds of its injection into the mother. The most rapid accumulation of F^{18} by the foetus occurred in the three minutes immediately following administration. Accumulation then continued at a slower rate for the duration of the experimental period.

Within eight minutes of the administration of the dose the rate of accumulation became constant (indicated by the straight line portion of the curve) and remained so for up to two and a half hours.

The amount of fluoride that accumulated in the foetus in the first three minutes was almost fifty percent of the amount that was present in one hour. This can be seen more clearly in Figure XVI, where the graphical mean is shown.

A moderate degree of positive correlation ($r = +.69$) existed between the administered dose (in μCi/gm of body weight) and percentage dose recovery but there was no correlation between foetal age and percentage dose recovery. (Appendix III).

FIGURE X

PERCENTAGE DOSE RECOVERY OF F^{18} IN WHOLE FOETUS

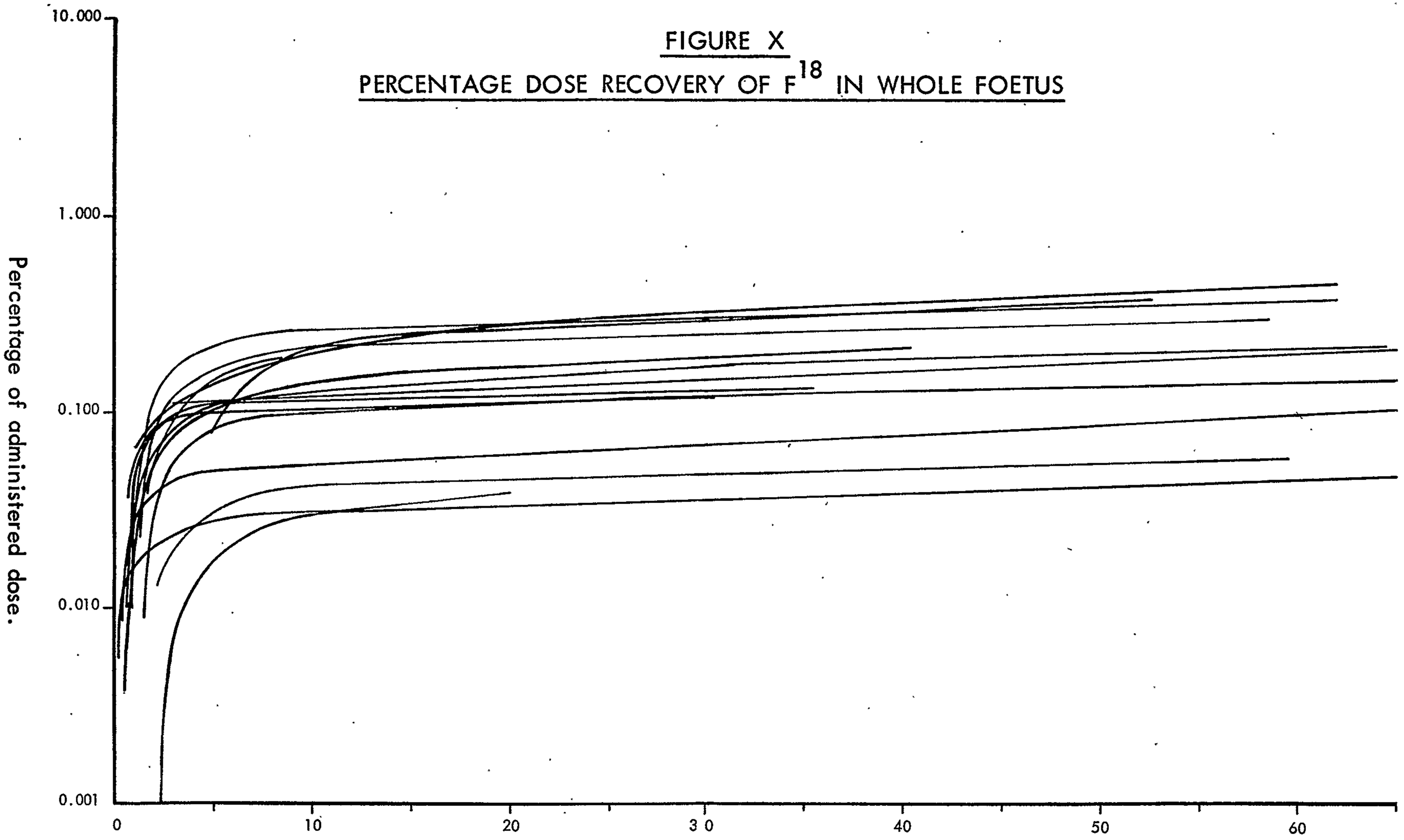


Fig. X shows the percentage of an administered dose of F^{18} in whole foetuses with time for a series of fifteen experiments.

2. WHOLE PLACENTA

Figure XI shows placental fluoride accumulation curves for twelve different studies, each consisting of a series of placentas.

The F^{18} levels varied but it was evident that there was a common pattern of accumulation.

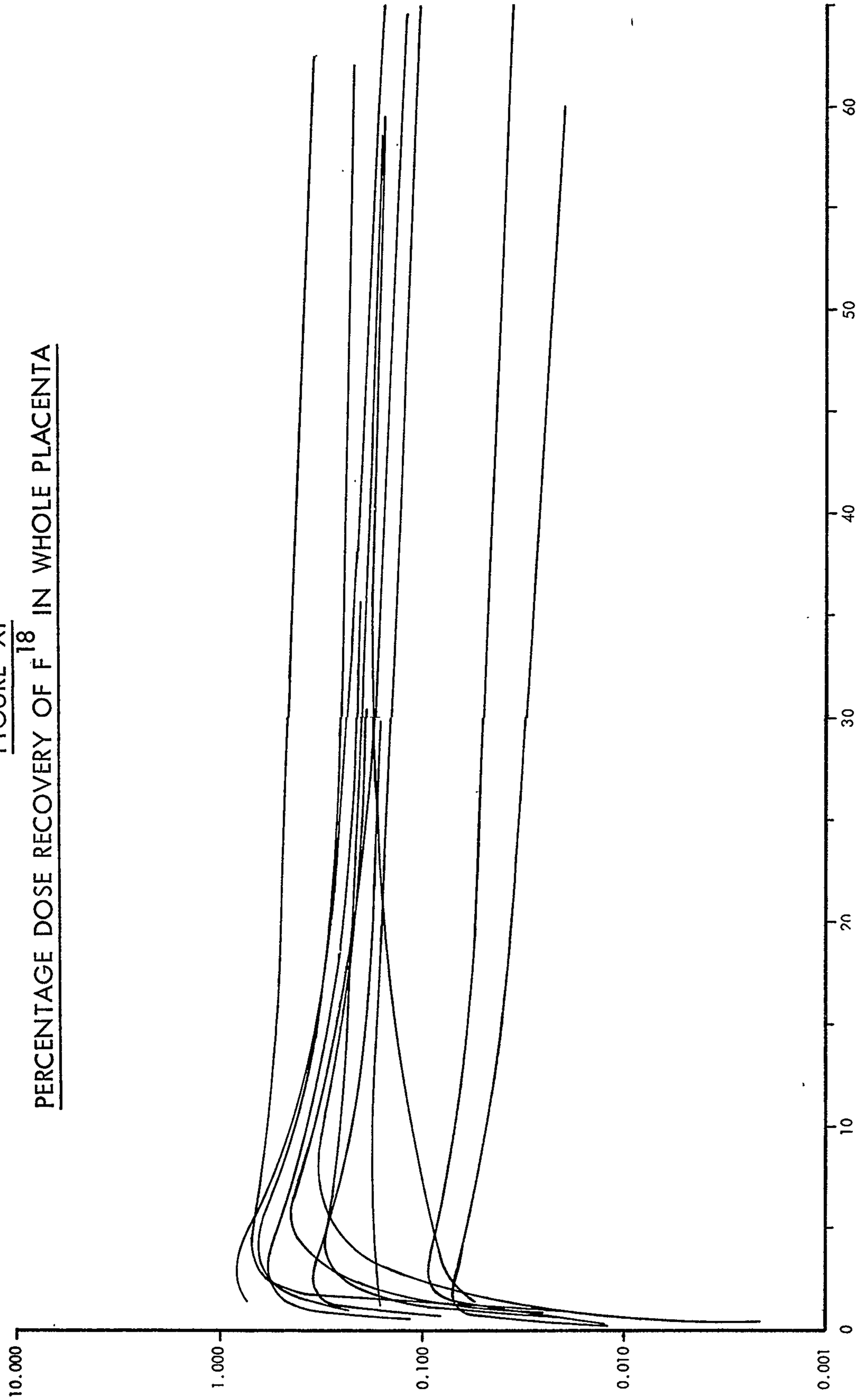
The accumulation of fluoride in the placenta reached a maximum (mean) value of 0.4 percent of the administered dose three minutes after the commencement of the injection. The content then began to decrease and continued to do so for as long as the study continued, the amount present in one hour being twenty-five percent of the maximum value.

The mean value is shown on Figure XVI.

No correlation was found between the magnitude of the dose and the percentage dose recovery or between foetal age and percentage dose recovery. (Appendix III)

FIGURE XI

PERCENTAGE DOSE RECOVERY OF F^{18} IN WHOLE PLACENTA



Percentage of administered dose.

Fig. XI shows the percentage of an administered dose of F^{18} in whole placentas with time for a series of twelve experiments.

3. MATERNAL BLOOD

The maternal blood clearance curves for F^{18} can be seen in Figure XII and the mean curve in Figure XVI.

Clearance was rapid and always followed the same pattern. The variation in absolute values between studies was slight and was not directly related to the magnitude of the administered dose.

The most rapid clearance occurred during the first three minutes. At this time 0.02 ml of blood contained only 0.025 percent of the administered dose. One hour after injection a sample of the same size contained only 0.0065 percent of the dose. For an average adult volume of 10 ml this indicates that slightly more than ninety percent of a single injected dose of F^{18} is cleared from the circulation within three minutes of its administration. The rate of clearance after this was much slower, approximately three percent of the dose remaining in the blood stream one hour after its administration.

The pattern of clearance from the blood of a non-pregnant adult female rat was essentially the same although fluoride levels in the blood of the latter were slightly lower than at corresponding times in the pregnant animal. In the non-pregnant rat it was found that the total fluoride present in the circulation thirty seconds after administration was no more than one third of the original dose.

These results are shown in Figure XIII and Appendix IV.

FIGURE XII

PERCENTAGE DOSE RECOVERY OF F^{18} IN MATERNAL BLOOD

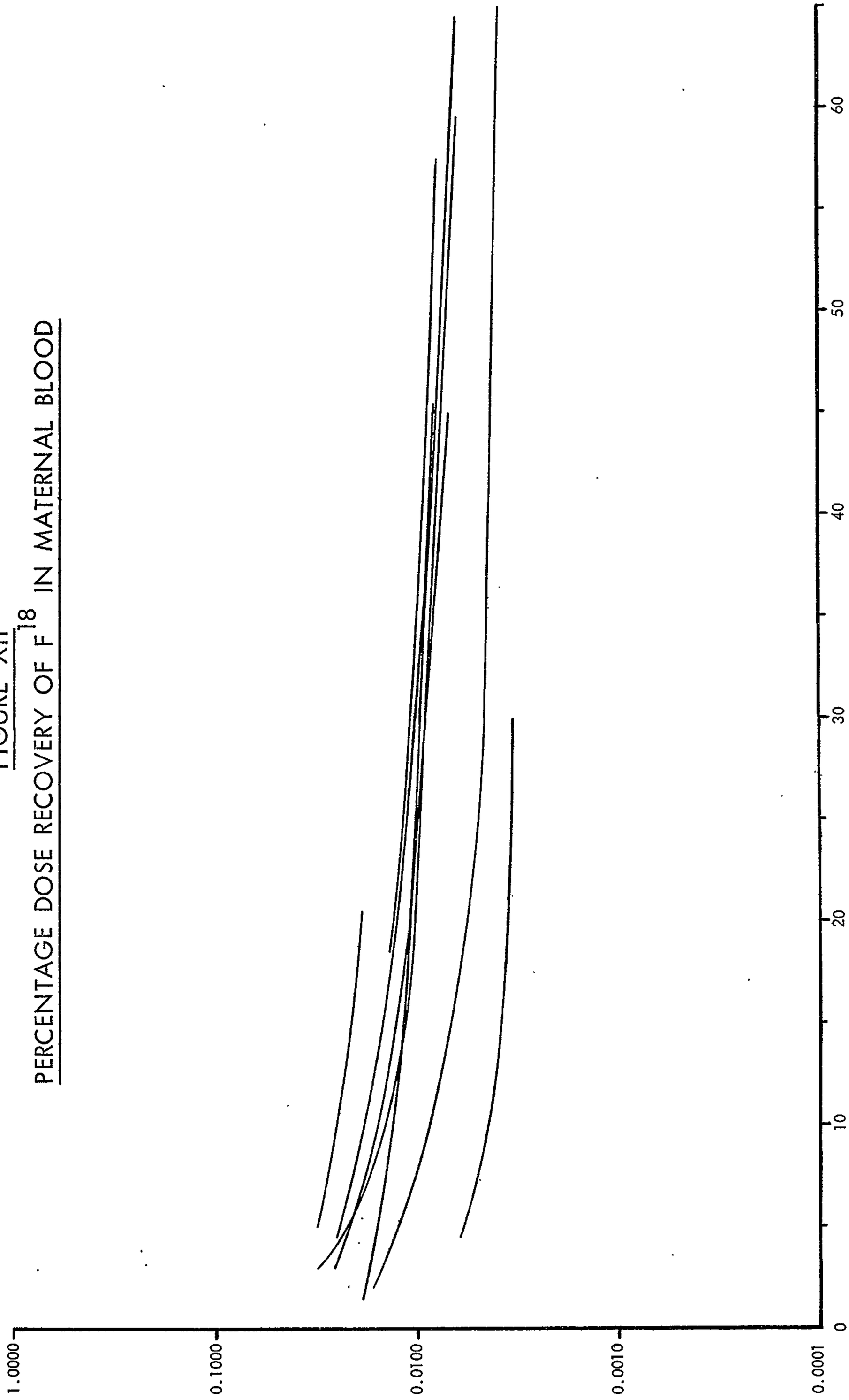
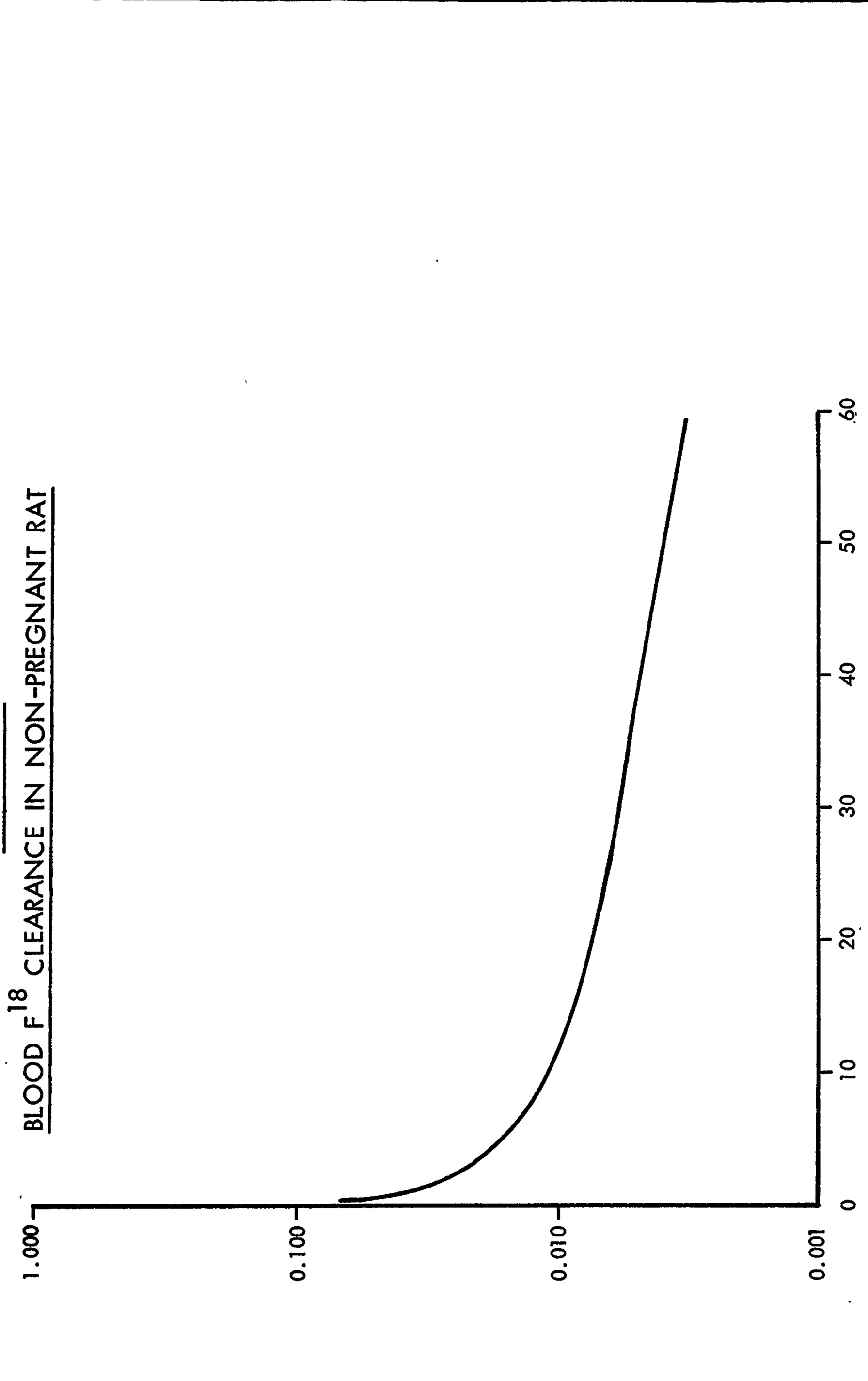


Fig. XII shows the percentage of an administered dose of F^{18} in 0.02 ml. of maternal blood with time.

Percentage of administered dose.

FIGURE XIII

BLOOD F^{18} CLEARANCE IN NON-PREGNANT RAT



Percentage of administered dose.

Fig. XIII shows the percentage of an administered dose of F^{18} in 0.02 ml. blood samples with time.

4. PLACENTAL BLOOD

The percentage dose recovery of fluoride in 0.02 ml of placental blood in seven experiments is shown in Figure XIV. The mean value for these studies is shown in Figure XVI.

It can be seen that the general pattern of accumulation was the same for all studies.

The blood fluoride content rose rapidly to a maximum two to three minutes after administration then began to decrease. One hour after injection the amount present was approximately thirty percent of the maximum value.

FIGURE XIV

PERCENTAGE DOSE RECOVERY OF F^{18} IN PLACENTAL BLOOD

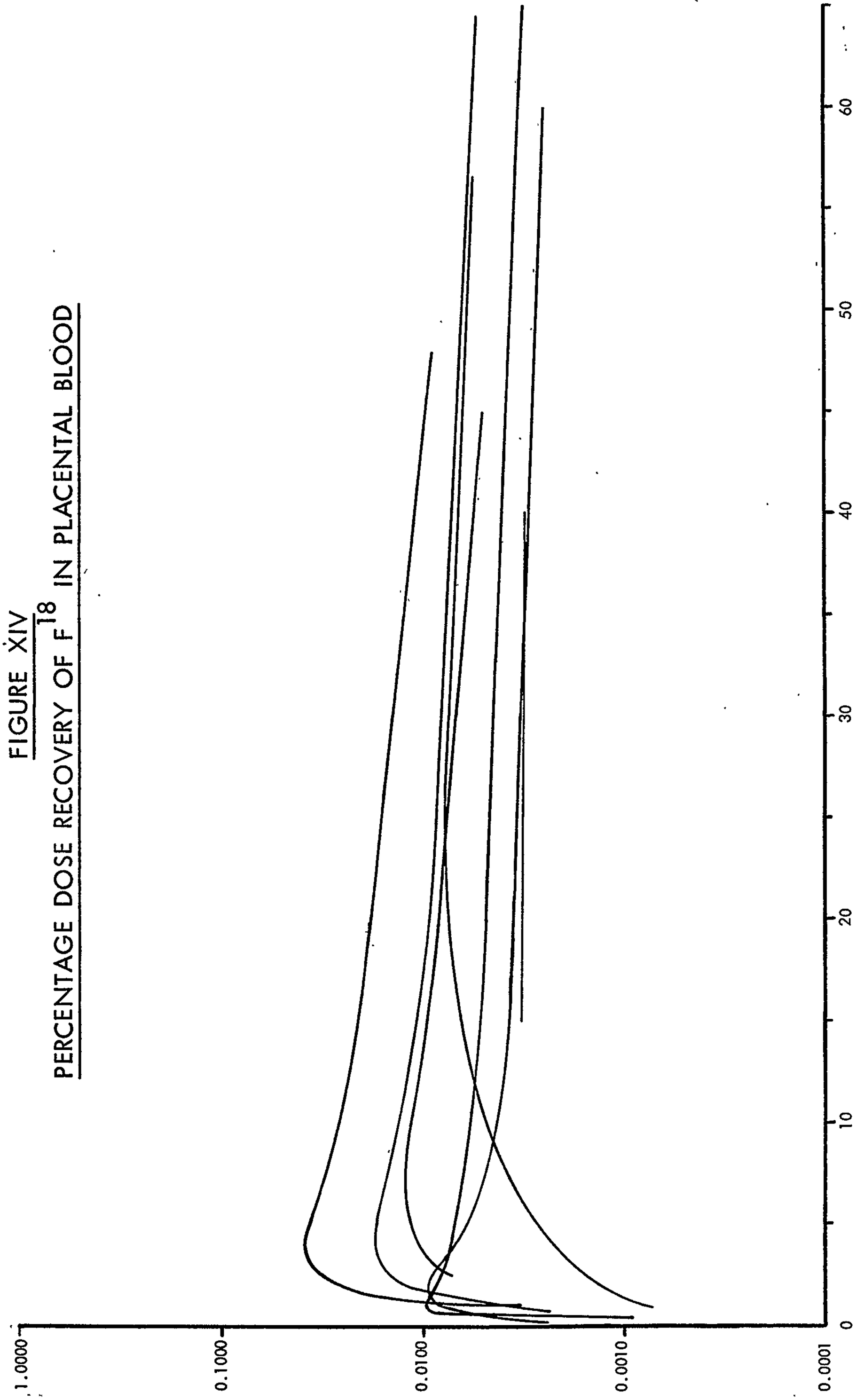


Fig. XIV shows the percentage of administered dose of F^{18} in 0.02 ml. of placental blood with time.

5. FOETAL BLOOD

The curves for five sets of results are shown in Figure XV and the mean in Figure XVI.

On examination of the values in Appendix II it can be seen that there was considerable variation in the amount of fluoride present in the blood of individual foetuses within a litter. The lines of best-fit do not indicate the extent of this variation and should not be considered as an unequivocal indication of foetal blood fluoride. However the fact that in the period from eight minutes to one hour after the injection these lines are parallel indicates that the trend is similar and that some reliability can be placed on this interpretation of the results.

The pattern of events is not clear but one of two possibilities may occur :

a. F^{18} reaches the foetal circulation within thirty seconds of its injection into the mother, immediately rises to its maximum value, then remains constant.

b. F^{18} quickly appears in the foetal blood at a high concentration but this rapidly decreases to reach a kind of equilibrium within eight minutes and then remains constant.

In either case the percentage of the administered dose present in 0.02 ml of blood was little more than 0.001 percent of the administered dose from eight minutes onwards.

The variation in values was not in any way related to the size of the dose.

FIGURE XV

PERCENTAGE DOSE RECOVERY CURVE OF F^{18} IN FOETAL BLOOD

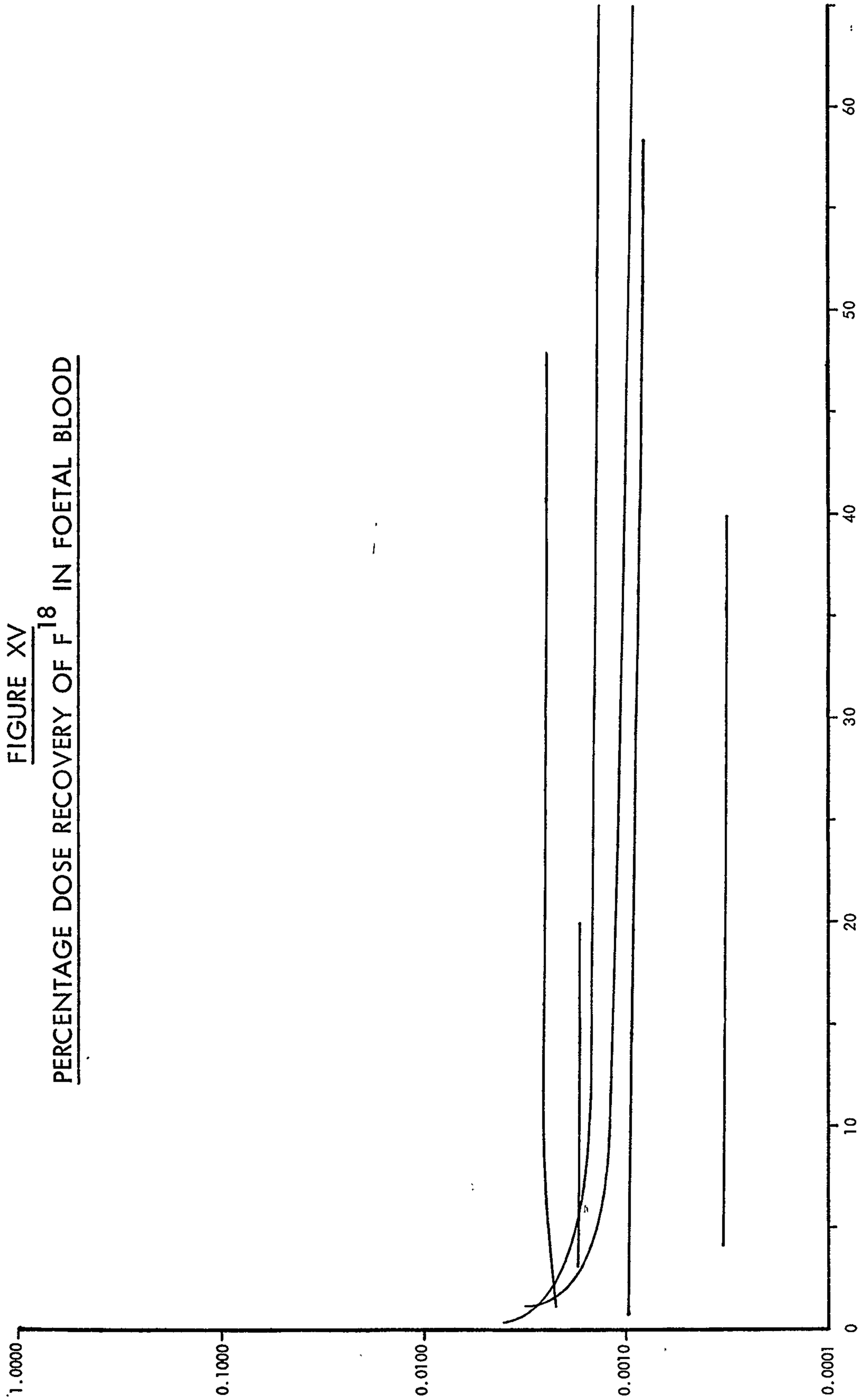


Fig. XV shows the percentage of an administered dose of F^{18} in 0.02 ml. of foetal blood with time.

6. AMNIOTIC FLUID

A negligible amount of F^{18} was present in the amniotic fluid. The results are shown below.

Time of Sample (min)	Percentage Dose Recovery
1	0.0035 *
3	nil
10	0.00007
20	0.00038 *

* Count was double background.

FIGURE XVI

MEAN PERCENTAGE DOSE RECOVERY CURVES

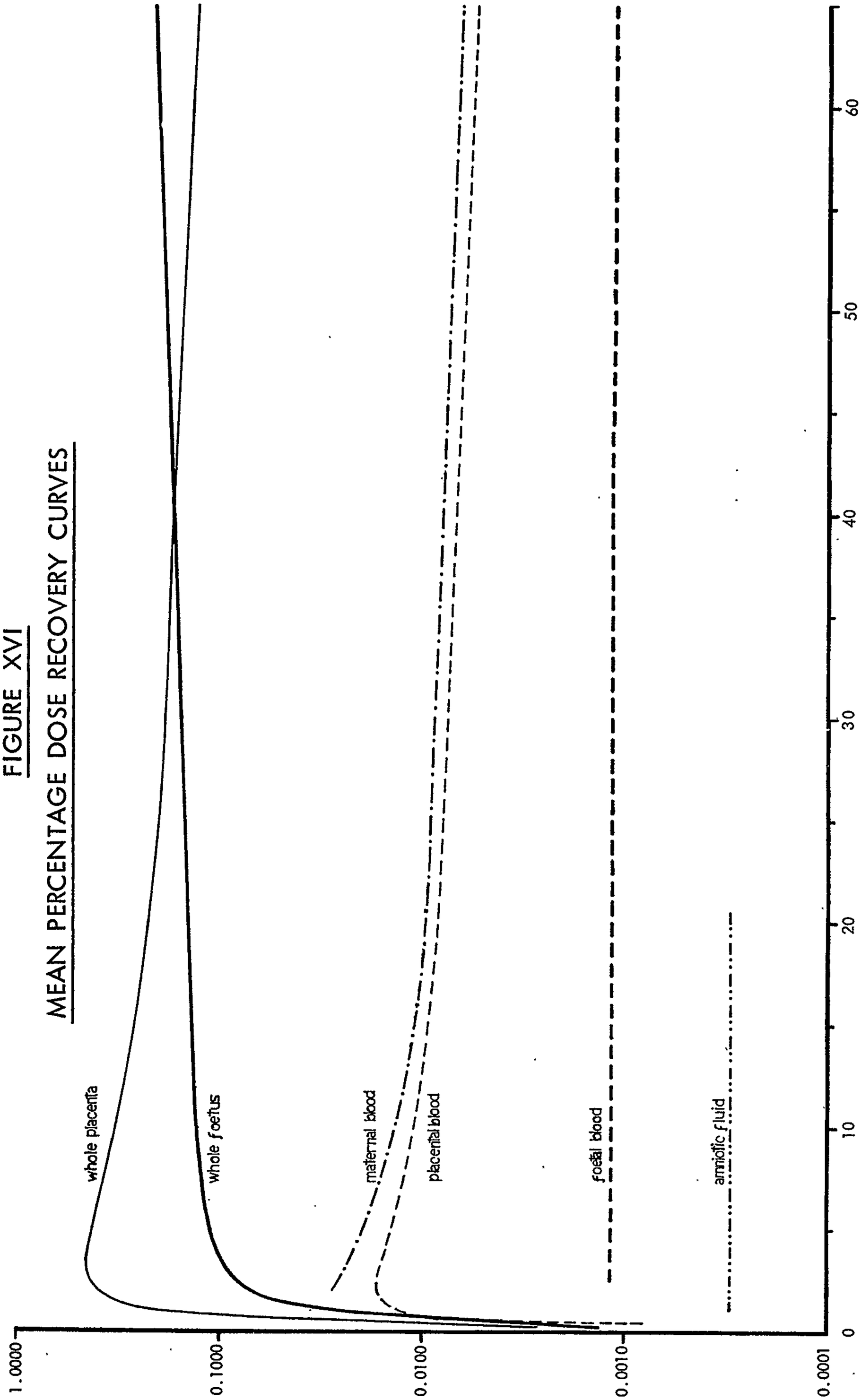


Fig. XVI shows the mean percentage of an administered dose of F^{18} in whole foetus and placenta and in 0.02 ml. samples of maternal, placental and foetal blood, and amniotic fluid with time for the series of experiments.

7. COMPARISONS

A composite figure was constructed showing the mean for each set of curves to enable ready comparison of the results. Certain relationships became apparent on examination of the data in Figure XVI, although strictly speaking quantitative comparisons cannot be made between samples of such different nature and size.

a. Whole Foetus and Whole Placenta

The period of most rapid accumulation of fluoride by the foetus coincided with that of the placenta but, whereas the placenta reached a maximum in three minutes and then decreased, the foetal content continued to increase for the whole study period. The placental content equalled the corresponding foetal content forty minutes after injection. At maximum placental accumulation (percentage recovery 0.46) the corresponding foetal content was a little less than one fourth of this.

b. Maternal, Placental and Foetal Blood

The close relationship of maternal and placental blood fluoride can be clearly seen on Figure XVI. Foetal blood fluoride content was much lower than these and did not exceed one tenth of the corresponding value over a period of one hour after administration. At the conclusion of the longest period of study, two and a half hours, the foetal blood fluoride level was only one fifth of the maternal level.

c. Whole Placenta, Placental Blood and Maternal Clearance

The curves for whole placenta and placental blood, being of essentially the same shape, indicated that the total placental content was a reflection of the placental blood content and depended on maternal clearance. There was therefore no accumulation in the placenta throughout the period of study.

d. Whole Foetus, Foetal Blood and Maternal Clearance

As long as fluoride was present in the maternal blood accumulation took place in the foetus but foetal blood fluoride was maintained at a constant level once its "equilibrium point" was reached. The rate of foetal accumulation decreased as maternal clearance proceeded.

8. COUNT VARIATION

There was little variation in the values obtained for the series of counts of the same foetus or placenta carried out to determine the range of count variation and the effect of change of sample position on the count.

The results are shown on Table VII.

TABLE VII

COUNT VARIATION

FOETUS				PLACENTA			
COUNT		PERCENTAGE DOSE RECOVERY		COUNT		PERCENTAGE DOSE RECOVERY	
NORMAL POSITION	TURNED 90°	NORMAL POSITION	TURNED 90°	NORMAL POSITION	TURNED 90°	NORMAL POSITION	TURNED 90°
3525	3391	.2271	.2185	2185	2893	.1814	.1864
3468	3539	.2235	.2281	2767	2636	.1783	.1699
3294	3472	.2123	.2237	2746	2741	.1770	.1766
3427	3455	.2208	.2226	2681	2632	.1728	.1696
3455	3342	.2226	.2154	2650	2760	.1708	.1779
3354	3506	.2161	.2259	2718	2730	.1751	.1759
3453		.2225		2756		.1776	
3365		.2168		2779		.1791	
				2663		.1716	
				2639		.1701	
				2685		.1730	
Mean	3418 3451	.2203	.2224	2661	2732	.1752	.1761
Combined Mean	3435	.2214 ± .0013		2697		.1755 ± 0011	
Range	3294 - 3539	.2123 -	.2281	2185 -	2893	.1696 -	.1864

DISCUSSION

Previously described in-vivo methods of investigating placental transfer of fluoride, and the findings which have been reviewed, indicate that the problem should be studied under conditions which more closely resemble the normal physiological environment.

Most of the data available has been obtained by chemical analysis of foetal tissues after administration of fluoride to the mother, as a dietary supplement, in food or water, through part, or all, of the period of gestation.

It is not possible, under such conditions, to observe continually the passage of fluoride in a single subject or experimental animal and without this it is difficult to make comparisons between species. There is also the problem of the detection of minute amounts of fluoride by microchemical methods.

The use of radioactive fluoride overcomes both of these difficulties but with the exception of the studies of Ericsson and Malmnäs[34] on humans and rabbits and Bawden et al[67], on sheep, an in-vivo radioisotope technique has not been used to study the placental transfer of fluoride. The consistent results obtained by these authors indicate that radioisotope studies are more likely to provide reliable information than chemical studies.

Bawden et al[6] were able to trace the transfer of F^{18} through the placenta of the pregnant sheep and could accurately determine its concentration in the maternal and foetal blood for a period of several hours. Ericsson and Malmnäs[34] were able to obtain only a few samples of foetal blood in humans but followed the transfer of F^{18} in the rabbit for a longer period.

The rat was chosen as the experimental animal for the present investigation because it has a placenta that is histologically similar to the human placenta, is a convenient size and has been used in many previous investigations of fluoride metabolism.

Satisfactory anaesthesia of the mother rat was readily obtained without affecting the foetuses [87] and the mother could be kept under anaesthesia for a considerable time. The longest experimental period used in this study was two and a half hours.

Intravenous injection of a known amount of F^{18} overcomes variations of intestinal absorption and allows all observations to be related to the original injected quantity.

The average quantity of F^{18} injected was approximately 0.3 MCi, which is equivalent quantitatively to 10^{-8} mg of fluoride. The maximum injected dose of 2.6 μCi/gm, or 2.6×10^{-11} mg/gm would be equivalent in the adult human to a concentration of 0.18×10^{-5} mg of fluoride. It is clear that the amount of fluoride administered was only a fraction of the normal physiological level.

The rate and pattern of maternal blood F^{18} clearance was examined in both the pregnant and non-pregnant rat. Clearance was found to be rapid, particularly in the first three minutes, during which time ninety percent of the administered fluoride disappeared from the blood stream. No significant difference was found between the pregnant and the non-pregnant rat, in either the rate or pattern of clearance. The F^{18} clearance curves for pregnant rats were similar to those obtained by Ericsson and Malmnäs [34] for pregnant women and rabbits, and by Bawden et al [6] for pregnant sheep.

The rapidity of clearance limits the amount of fluoride potentially available to the foetus. Irrespective of the intake, high concentrations are not available for more than a few minutes. It was found in this study however that even with a low initial quantity of F^{18} in the maternal blood, some of this could be detected in the foetal circulation almost immediately after injection, despite the rapidity of the maternal clearance. Its presence appeared to be related to the maternal circulation time.

The accumulation of F^{18} by the foetus commenced as soon as F^{18} was available from the circulation. The most rapid accumulation occurred during the first three minutes and then the rate of accumulation became slower and appeared to become

constant. After two hours the calculated total amount of F^{18} in a litter in two specific examples was found to be 0.7 and 1.4 percent of the original dose. This agrees with that found by Bawden et al [7] in the sheep. The period of rapid accumulation by the foetus, appears to coincide with the period of rapid clearance in the maternal blood. If this is more than chance it could indicate that the change in rate of foetal uptake is related to a decrease in F^{18} concentration in foetal blood, corresponding to the clearance of F^{18} from maternal blood.

It was difficult to determine what changes in the F^{18} concentration of foetal blood occurred during the first eight minutes after injection of the mother because of considerable variation of F^{18} levels over this period. In only two experiments the pattern was similar to a maternal clearance curve, but any such similarity was not observed by Bawden et al [6] or Ericsson and Malmnäs [34].

After the first eight minutes the foetal blood F^{18} level appeared to be stable until the end of the experimental period, as long as two and a half hours. Some fluctuation in blood F^{18} concentration between foetuses was observed during this period. This could have been due to actual variations or could have been the result of dilution, as it was difficult to be certain that the blood samples were completely free of tissue fluid and this may have affected the observed F^{18} values. Consequently the observed intermittent low values may not be valid. This would not alter the characteristics of the foetal blood curve, but, since a line of best fit was drawn for each experiment, the mean curve finally obtained may have been slightly lower than the true value. This would not, however, substantially alter the relationship between foetal blood F^{18} and maternal blood F^{18}.

The concentration of F^{18} found in foetal blood was always much less than that in the corresponding maternal blood and, for the major portion of the experimental period, the ratio was approximately 1:10. The highest ratio of 1:5 was obtained two and a half hours after injection. Bawden et al [6] and Ericsson and Malmnäs [34] found a general relationship between maternal and foetal blood F^{18} concentrations similar to that found in the present study.

If the placenta were freely permeable to fluoride, equal concentrations of F^{18} in the foetal and maternal blood at all times would be expected unless foetal blood clearance was even more efficient than maternal clearance. The findings indicate that the placenta is not freely permeable to fluoride but that there is some restriction on transfer. As pointed out by Bawden et al [7], the fact that foetal blood F^{18} is lower at all times than maternal could be a result of those aspects of circulation dynamics involving foetal placental blood flow and maternal placental exchange, as well as a restriction by the placenta.

The experimental results do not confirm the observations of some investigators that maternal and foetal blood contain not only equal concentrations of fluoride [46 48 59], but that foetal blood has a higher concentration than maternal [48 58 115]. The findings of the present study indicate that foetal blood F^{18} concentration has an upper limit which remains constant as long as the maternal concentration exceeds this level.

Gedalia et al[48] found that when the intake of fluoride was low human foetal blood contained more fluoride than corresponding maternal blood but that when the intake was high this relation was reversed. They also found that the foetal cord blood fluoride concentration remained constant even though the maternal blood concentration changed. This fact tends to substantiate the theory that foetal blood fluoride concentration has an upper limit. It indicates in addition that in humans there is some mechanism operating to maintain this concentration even if the maternal blood concentration is lower.

As already pointed out the levels of fluoride injected in the present study are much lower than the amount of fluoride normally ingested by humans and are only a fraction of the fluoride values of the low intake group examined by Gedalia et al[48]. Their results are not substantiated by the findings of this study. However, the mechanisms acting in rats are not necessarily the same as those in humans. The similarity of the findings by Bawden et al [6 7] for the placental transfer of F^{18} in sheep to the findings obtained in this study for transfer in the rat indicate that transfer of

fluoride through the syndesmochorial placenta is the same as through the haemochorial placenta.

It was difficult to determine the effect of gestation age on placental permeability or foetal uptake from the observations of this study. There is a possibility that gestation age would have some effect because it has been shown that placental permeability to certain substances changes with age. There was no evidence to show that there was any increase in placental permeability as gestation age increased. The amount of fluoride that was taken up by the foetus in any particular period was no higher in a twenty day foetus than in a seventeen day foetus, even though foetal size was greater. Neither was there any relation between foetal blood fluoride concentration and foetal age.

It should be noted that the removal of each successive foetus does produce some changes in the experimental conditions, however, the effect of these changes was not investigated.

Limitations in transfer do not necessarily indicate storage by the placenta because no accumulation of F^{18} was found during the period studied and in fact the total placental F^{18} content was directly proportional to the F^{18} content of placental blood, which was not significantly different from maternal blood concentration. In both whole placenta and placental blood the maximum concentration was reached approximately three minutes after administration, probably indicating the time required for complete maternal placental exchange. The F^{18} content of the whole placenta then decreased in direct proportion to placental blood.

Ericsson and Malmnäs[34] and Ericsson and Hammarström[33] have also reported that accumulation of F^{18} in the placenta does not occur, except in areas where calcium salts have been deposited. Comparison of placental fluoride with blood fluoride by other investigators is only useful when the results are expressed on the same weight or volume basis and some of the high values reported may be the result of accumulation of fluoride in areas of calcium deposition.

The mechanism of placental transfer of fluoride obviously needs further investigation, particularly the relationship between maternal and foetal blood fluoride concentrations. The results presented in this thesis demonstrate that while placental transfer in the rat occurs quickly even at very low levels, there is some degree of restriction imposed by the placenta and although accumulation of fluoride by the placenta was not found, there was some evidence of a regulatory mechanism whereby the placenta influenced the proportional concentration of F^{18} in the maternal and foetal blood.

SUMMARY

A review of experimental work on both humans and animals indicates that the body has a very efficient fluoride balance mechanism. Upon absorption, clearance from the blood stream is rapid, so that the blood fluoride content is kept at a constant level and any excess fluoride not excreted is deposited in the skeleton and, to a much lesser extent, in the teeth. During pregnancy fluoride passes through the placenta and has been found in foetal tissues of humans and a variety of animals.

A significant degree of resistance to dental caries results from the deposition of fluoride in the teeth, but the amount of resistance that can be attributed to prenatal fluoride has not been clearly established. By studying placental transfer of fluoride the effect of prenatal fluoride on caries resistance could be clarified.

As with most elements passage through the placenta is a two way process. Past evidence in general indicates that fluoride concentration is higher in maternal than in foetal blood and that accumulation of fluoride by the placenta does not occur, except in relation to areas where calcium salts have been deposited. The fluoride content of foetal bones and teeth increases with increased maternal intake but not in direct proportion. There appears to be only slight transfer in animals when there is less than 10 ppm in the diet or drinking water but transfer in humans occurs at much lower levels.

A method of studying the placental transfer of fluoride in the rat using the radioisotope of fluorine F^{18}, under conditions approaching the physiological normal has been developed.

Sixteen pregnant rats were used in the study at stages of gestation varying from seventeen to twenty-one days. After foetal externalization under anaesthesia radioactive fluoride was injected into the mother. The injected dose of radioisotope never exceeded 2.6μCi per gram of body weight. The foetuses and placentas were removed at intervals over periods extending up to two and a half hours and 0.02 ml samples of maternal, placental and foetal blood and amniotic fluid were obtained.

All F^{18} values were corrected for decay to the time of commencement of the injection and then expressed as a percentage of the administered dose. Although some problems were encountered in obtaining foetal blood samples, the technique was in general satisfactory.

No significant difference existed between the rate and pattern of blood clearance for F^{18} in the pregnant or non-pregnant rat. Within three minutes of its administration approximately ninety percent of the fluoride was cleared from the circulation. The placental blood content was found to be very similar but slightly lower than maternal blood within two minutes of injection of F^{18}. The foetal blood however had a much lower fluoride content and appeared to be stable from the eighth minute to the end of the experimental period. In the first eight minutes very variable results were obtained. This variation and the possible inaccuracies caused by difficulties in sampling prevent any definite conclusions being drawn for this period (0 to 8 minutes after injection).

The accumulation of fluoride by the whole foetus occurred throughout the entire experimental period and the decreasing rate of foetal accumulation appeared to be coincident with maternal clearance. Percentage dose recovery in the whole foetus was in general related to the total amount of F^{18} injected but not to foetal age investigated. (17 - 21 days).

The F^{18} content of the whole placenta was directly proportional to the placental blood fluoride level and appeared related to maternal clearance not foetal requirements. There was no placental accumulation over the experimental period.

The findings indicated that the fluoride reaching the foetus was limited by rapid maternal homeostasis and regulated by the placenta.

CONCLUSIONS

The maternal blood clearance of F^{18} by the rat is rapid and efficient. Ninety percent is cleared within the first three minutes after administration.

Using very low levels of fluoride as F^{18} there is rapid transfer of fluoride to the foetal blood and foetus.

The foetal blood F^{18} concentration varies between one tenth and one fifth of the concentration of F^{18} in the corresponding maternal blood.

The foetal blood concentration appears to remain constant with time despite the decrease in maternal blood fluoride concentration, for periods up to two and a half hours.

Accumulation of F^{18} in the foetus occurred throughout the entire experimental period.

The placental blood F^{18} concentration was only slightly lower than that of the maternal blood at all times and there was no accumulation of F^{18} in the whole placenta.

The placenta is not freely permeable to fluoride and appears to have a regulatory function and in fact restricts the amount of fluoride that reaches the foetus.

The use of radioisotope and foetal externalization techniques is a satisfactory method of investigating the placental transfer of fluoride in the rat and could be used subsequently to determine the degree of restriction of fluoride transfer by the placenta under variable experimental conditions.

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Am.J.Pub.Hlth., 47:848-851, July 1957.

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Urinary fluoride levels associated with use of fluoridated waters.
Pub.Health Rep., 71:767-772, Aug. 1956.

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Pub.Health Rep., 73:732-740, Aug. 1958.

APPENDIX I

RADIATION DETECTING EQUIPMENT

1. Type Used for Blood Samples

EKCO Scintillation Counter Type N664A

using a $1\frac{1}{2}$ inch plastic phosphor

EKCO Automatic Scaler Type N530G

2. Type Used for Whole Foetus and Placenta

Field Scintillation Counter Type N618A

using 1 inch x 1 inch thallium activated

sodium iodide crystal

Shield - Phillips castle Type PW 4124/00

EKCO Automatic Scaler Type N610A

APPENDIX II

SUMMARY ALL RESULTS FOR PERCENTAGE DOSE RECOVERY OF F^{18}

Time sample min.sec.	Percentage Dose Recovery				
	Whole foetus	Whole placenta	0.02ml foetal blood	0.02ml placental blood	0.02ml maternal blood
10	0.0055	0.0126	0.0066	0.0024	-
30	0.0127	0.0295	0.0024	0.0052	-
2	0.0200	0.0644	0.0012	0.0092	0.0166
10	0.0314	0.0385	0.0014	0.0031	0.0087
20 30	0.0335	0.0311	0.0006	0.0044	0.0053
40	0.0381	0.0299	0.0033	0.0023	0.0042
60	0.0463	0.0174	0.0012	0.0030	0.0041
80	0.0806	0.0184	0.0009	0.0016	0.0036
105	0.0590	0.0122	0.0006	0.0017	0.0022
121	0.0715	0.0104	0.0008	0.0013	-
138	0.0777	0.0154	0.0009	0.0016	0.0016
150	0.0640	0.0054	0.0003	0.0010	0.0015
Preoperative Weight = 425 gm. Foetal age = 18 days Dose = .203 MCi = 0.48 μCi/gm.					
24	0.0089	0.0249	-	0.0009	-
1	0.0714	0.0723	0.0044	0.0093	-
2 30	0.0299	0.0552	0.0011	0.0072	-
3	-	-	-	-	0.0314
4	0.0321	0.0876	0.0019	0.0071	-
6 30	0.0368	0.0789	0.0008	-	-
11 30	-	-	-	-	0.0125
20	0.0316	0.0489	0.0026	0.0048	-
20 30	-	-	-	-	0.0109
59 30	-	-	-	-	0.0061
60	0.1259	0.0500	0.0008	0.0032	-
90	0.1247	0.0304	0.0006	0.0037	-
90 30	-	-	-	-	0.0042
121	0.1442	0.0362	0.0005	0.0027	-
Preoperative Weight = 495 gm. Foetal age = 20 days Dose = .200 MCi = 0.40 μCi/gm.					

APPENDIX .II (Cont'd.)

Percentage Dose Recovery

Time sample min.sec.	Whole foetus	Whole placenta	0.02 ml foetal blood	0.02 ml placental blood	0.02 ml maternal blood
45	0.5193	0.0647	0.0010	0.0008	-
1 15	0.0290	0.0289	0.0001	-	-
1 30	0.0238	0.0202	-	0.0010	-
6 30	0.1626	0.2660	0.0010	-	-
11 30	0.2663	0.2918	0.0008	0.0043	-
18 30	-	-	-	-	0.0132
26 30	0.1982	0.1794	0.0010	0.0076	-
27 30	-	-	-	-	0.0110
41 30	0.2397	0.1783	0.0005	0.0073	-
43 30	-	-	-	-	0.0082
56 30	0.2603	0.2257	0.0007	0.0055	-
57 30	-	-	-	-	0.0078
58 30	0.3207	0.2024	0.0009	-	-
Preoperative Weight = 289 gm. Foetal age = 18 days Dose = 0.380 MCi = 1.31 μCi/gm.					
45	0.0266	0.0382	-	0.0024	-
1	0.0264	0.0244	-	0.0039	-
1 30	0.0771	0.0794	-	0.0041	-
4 30	0.1156	0.3993	-	0.0170	0.0247
14 30	0.1113	0.2256	-	0.0109	0.0155
24 30	0.1173	0.1579	-	0.0077	0.0113
34 30	0.1811	0.1611	-	0.0077	0.0091
44 30	0.1953	0.1848	-	0.0080	0.0077
54 30	0.2193	0.1298	-	0.0055	0.0072
64 30	0.2092	0.1009	-	0.0052	0.0063
Preoperative Weight = 349 gm. Foetal age = 17 days Dose = 0.232 MCi = 0.67 μCi/gm.					

APPENDIX II (Cont'd.)

Time sample min.sec.	Percentage Dose Recovery				
	Whole foetus	Whole placenta	0.02 ml foetal blood	0.02 ml placental blood	0.02 ml maternal blood
1	0.0662	0.2356	0.0023	0.0033	-
4 30	0.1091	0.9766	0.0014	0.0378	-
5	-	-	-	-	0.0313
9	0.1507	0.3211	0.0021	0.0248	-
9 30	-	-	-	-	0.0258
14	0.3018	0.3888	0.0038	0.0217	-
14 30	-	-	-	-	0.0213
20	0.2416	0.2340	0.0023	0.0181	-
20 30	-	-	-	-	0.0184
45	0.4504	0.2375	0.0025	0.0100	-
48	0.4509	0.2690	0.0024	0.0080	-
62	0.3655	0.2416	-	-	-

Preoperative Weight = 305 gm Foetal age = 18 days Dose = 0.541 MCi = 1.77 μCi/gm.

1	*	-	0.1751	-	-	-
3		0.1143	0.1458	0.0018	0.0077	0.0257
3	*	0.1060	0.1715	-	-	-
10		0.0858	0.1650	0.0011	0.0113	0.0150
10	*	0.1349	0.1825	-	-	-
20		0.1538	0.1900	0.0019	0.0088	0.0105
20	*	0.1292	0.1475	-	-	-
30		0.1458	0.1219	-	0.0070	0.0090
45		0.1573	0.1306	-	0.0044	0.0065
75		0.2443	0.1208	-	-	-
79	30*	0.2140	0.0855	-	-	-

* foetus left in embryonic sac.

Preoperative Weight = 453 gm Foetal age = 19 days Dose = 0.166 MCi = 0.37 μCi/gm

APPENDIX II (Cont'd.)

Time sample min.sec.	Percentage Dose Recovery				
	Whole foetus	Whole placenta	0.02 ml foetal blood	0.02 ml placental blood	0.02 ml maternal blood
1 30	0.0099	0.7375	-	-	0.0184
5 30	0.1237	0.5396	-	-	0.0157
10 30	0.0861	0.5975	-	-	0.0129
15 30	0.0969	0.2173	-	-	0.0114
25 30	0.1112	0.3014	-	-	0.0118
35 30	0.1482	0.1608	-	-	0.0094
45 30	0.1331	0.1745	-	-	0.0068
56	0.1357	0.1383	-	-	-
65 30	0.1317	0.2550	-	-	-
70 30	0.0222	0.1356	-	-	-
Preoperative Weight = 384 gm		Foetal age = 17 days		Dose = 0.136 MCi = 0.35 μCi/gm	
30	-	0.0379	-	-	-
4 30	-	0.0369	0.0003	-	-
5	-	-	-	-	0.0050
8	-	0.0274	0.0004	-	-
8 30	-	-	-	-	0.0047
15	-	0.0935	0.0004	0.0028	-
16	-	-	-	-	0.0038
25	-	0.0878	-	-	-
30	-	-	-	-	0.0033
31	-	0.1399	0.0002	0.0034	-
40	-	0.0752	0.0003	0.0031	-
Preoperative Weight = 316 gm		Foetal age = 18 days		Dose = 0.580 MCi = 1.84 μCi/gm	

APPENDIX II (Cont'd.)

Time sample min.sec.	Percentage Dose Recovery				
	Whole foetus	Whole placenta	0.02 ml foetal blood	0.02 ml placental blood	0.02 ml maternal blood
45		0.0404	0.2754		
1		0.0114	0.1146		
1 15		0.0858	0.3674		
1 30		0.0951	0.5589		
2 30		0.1181	0.5296		
4 30		0.1413	0.3753		
7 30		0.0611	0.2621		
10 30		0.0831	0.3971		
15 30		0.0663	0.2968		
20 30		0.1475	0.2732		
30 30		0.1573	0.2959		
35 30		0.0904	0.1415		
Preoperative Weight = 325 gm		Foetal age = 17 days		Dose = 0.126 MCi = 0.39 μCi/gm	
30		0.0037	0.0021		
45		0.0144	0.0311		
1		0.0065	0.0096		
1 30		0.0470	0.0816		
3		0.0791	0.1466		
4		0.0558	0.0828		
7 24		0.1367	0.3636		
10 24		0.1255	0.2176		
15 24		0.1743	0.2395		
20 24		0.1768	0.1805		
30 24		0.1755	0.2464		
40 24		0.2253	0.1799		
Preoperative Weight = 316 gm		Foetal age = 17 days		Dose = 0.140 MCi = 0.44 μCi/gm	

APPENDIX II (Cont'd.)

Time sample min.sec.	Percentage Dose Recovery				
	Whole foetus	Whole placenta	0.02 ml foetal blood	0.02 ml placental blood	0.02 ml maternal blood
45		0.0133	0.0306		
3 30		0.0209	0.0793		
5 30		0.0224	0.0999		
10 30		0.0404	0.1221		
15 30		0.0533	0.1584		
20 30		0.0408	0.1148		
25 30		0.0451	0.1808		
30 30		0.0457	0.2242		
35 30		0.0527	0.1754		
40 30		0.0477	0.1113		
45 30		0.0533	0.1712		
59 30		0.0601	0.1590		
Preoperative Weight = 292 gm		Foetal age = 17 days		Dose = 0.446 MCi = 1.53 μCi/gm	
45		0.0449	0.0801		
1		0.0343	0.0386		
1 15		0.1043	0.3400		
1 30		0.0648	0.1600		
2 30		0.1068	0.2203		
5 30		0.0993	0.2302		
8 30		0.0818	0.2047		
10 30		0.0319	0.1421		
15 30		0.1022	0.1979		
20 30		0.1133	0.2278		
30 30		0.1132	0.1819		
Preoperative Weight = 295 gm		Foetal age = 17 days		Dose = 0.147 MCi = 0.50 μCi/gm	

APPENDIX II (Cont'd.)

Time sample min.sec.	Percentage Dose Recovery				
	Whole foetus	Whole placenta	0.02 ml foetal blood	0.02 ml placental blood	0.02 ml maternal blood
45		0.0202	0.0345		
1 15		0.0200	0.0202		
2 15		0.0016	0.0004		
3 30		0.2451	0.6662		
16 30		0.2368	0.5449		
26 30		0.3988	0.4854		
41 30		0.2195	0.3750		
52 30		0.3018	0.3319		
61 30		0.4329	0.3871		
62 30		0.1798	0.2792		
Preoperative Weight = 283 gm Foetal age = 19 days Dose = 0.358 MCi = 1.26 μCi/gm					
16		-			
1		-			
2 56		0.0017			
3		0.0007			
4		0.0115			
10		0.0362			
15		0.0281			
20		0.0468			
Preoperative Weight = 438 gm Foetal age = 12 days Dose = 0.298 MCi = 0.68 μCi/gm					

APPENDIX II (Cont'd.)

Time sample min.sec.	Percentage Dose Recovery				
	Whole foetus	Whole placenta	0.02 ml foetal blood	0.02 ml placental blood	0.02 ml maternal blood
1 30			0.0418		
3 30			0.0788		
5 30			0.1547		
8 30			0.1890		
20 30			0.0794		
Preoperative Weight = Not known Foetal age = 18 days Dose = .0215 MCi					
5			0.0827		
8 30			0.1661		
11			0.2725		
15			0.2087		
25			0.3685		
30			0.2016		
40			0.3282		
52 30			0.4946		
Preoperative Weight = Not known Foetal age = 19 days Dose = 1.174 MCi					

APPENDIX III

RELATIONSHIP OF STUDY VARIATION TO DOSE SIZE AND FOETAL AGE

Dose Weight		Dose Weight	Foetal Age	Percentage Dose Recovery Approximate 10 Minute Sample				
MCi	gm	μCi/gm	days	Whole Foetus	Whole Placenta	Mat. Blood	Plac. Blood	Foetal Blood
0.203	425	0.48	18	0.0314	0.0385	0.0087	0.0031	0.0014
.200	495	0.40	20	.0368	.0789	.0125	-	.0008
.380	289	1.31	18	.2663	.2918	-	.0043	.0008
.232	349	0.67	17	.1113	.2256	.0155	.0109	-
.541	305	1.77	18	.1507	.3211	.0258	.0248	.0021
.166	453	0.37	19	.0858	.1650	.0150	.0113	.0011
.136	384	0.35	17	.0861	.5975	.0129	-	-
.580	316	1.84	18	-	.0274	.0047	.0028	.0004
.126	325	0.39	17	.0831	.3971	-	-	-
.140	316	0.44	17	.1255	.2176	-	-	-
.446	292	1.53	17	.0404	.1221	-	-	-
.147	295	0.50	17	.0319	.1421	-	-	-
.358	283	1.26	19	.2368	.5449	-	-	-
.298	438	0.68	12	.0362	-	-	-	-
Mean								
.282	354	0.85						

Average number in Litter = 10.

APPENDIX IV

F^{18} CLEARANCE FROM BLOOD OF NON-PREGNANT FEMALE RAT

Time Sample taken min. sec.	Percentage Dose Recovery
24	0.0664
36	0.0571
46	0.0447
1	0.0375
1 30	0.0316
1 45	0.0306
2	0.0256
2 30	0.0243
3	0.0211
3 30	0.0199
4	0.0194
4 30	0.0174
5	0.0161
6	0.0145
7	0.0135
8	0.0126
9	0.0116
10	0.0114
15	0.0085
20	0.0071
25	0.0062
30	0.0060
45 30	0.0041
60	0.0031